Dendritic Cells in *Helicobacter Pylori*-Specific Immune Tolerance and Asthma Protection

DISSERTATION

ZUR

ERLANGUNG DER NATURWISSENSCHAFTLICHEN DOKTORWÜRDE

(DR. SC. NAT.)

VORGELEGT DER

MATHEMATISCH-NATURWISSENSCHAFTLICHEN FAKULTÄT

DER

UNIVERSITÄT ZÜRICH

VON

MATHIAS OERTLI

VON

ENNENDA, GL

PROMOTIONSKOMITEE:

PROF. DR. ANNE MÜLLER (VORSITZ UND LEITUNG DER DISSERTATION)

PROF. DR. MARIES VAN DEN BROEK

PROF. DR. BURKHARD BECHER

PD. DR. LUBOR BORSIG

ZÜRICH, 2012

I. SUMMARY

Persistent infection with the gastric bacterial pathogen *Helicobacter pylori* (*H. pylori*) causes gastritis and predisposes carriers to a high gastric cancer risk, but has also been linked to protection from allergic, chronic inflammatory and autoimmune diseases. The beneficial consequences of chronic *H. pylori* infection have been proposed to result from the pathogen's immunomodulatory properties. We show here that *H. pylori* inhibits the LPS-induced maturation of dendritic cells (DCs) as assessed by CD80/CD86 upregulation and IL-12 secretion, and re-programs DCs towards a tolerance-promoting, semi-mature state. DCs that have been exposed to *H. pylori in vitro* or *in vivo* fail to induce T cell effector functions, and instead efficiently induce FoxP3 expression and suppressive properties in naive T cells. The experimental depletion of DCs breaks *H. pylori*-specific tolerance and results in improved control of the infection, but also in more severe immunopathology. Low-dose LPS administration overrides the manipulation of DCs by *H. pylori in vitro* and *in vivo*. Moreover, DCs infiltrating the gastric mucosa of human *H. pylori* carriers retain a semi-mature $CD80^{low}CD86^{low}$ state. Mechanistically, the tolerogenic activity of *H. pylori*-experienced DCs is shown to require IL-18 *in vitro* and *in vivo*. DC-derived IL-18 directly acts on T cells to drive their conversion to regulatory T cells (Treg). Additionally, two bacterial factors, γ-glutamyl transpeptidase (GGT) and vacuolating cytotoxin (VacA) are shown to contribute critically to the tolerization of DCs by *H. pylori*. Isogenic mutants lacking GGT or VacA fail to drive DC tolerization and consequently, these DCs fail to drive FoxP3 expression in naive T cells. Both mutants fail to colonize mice at wild type levels, induce stronger Th1 and Th17 responses and/or trigger more severe gastric pathology.

Moreover, $CD4^{+}CD25^{+}$ Tregs from infected wild type mice but not *Il-18*$^{-/-}$ or *Il-18r*$^{-/-}$ mice protect from clinical and histopathological manifestations in an experimental model of asthma. Additionally, asthma protection is lost, if the mice were infected with Δ*vacA* mutant prior to allergen challenge, underscoring the importance of this bacterial protein in skewing the immune system towards tolerance.

Overall, our results indicate that tolerogenic re-programming of DCs ensures the persistence of *H. pylori* and protects against asthma in a process that requires IL-18 and the two bacterial factors GGT and VacA.

II. ZUSAMMENFASSUNG

Eine persistente Infektion mit dem bakteriellen Magenpathogen *Helicobacter pylori* führt zu chronischer Gastritis und bringt ein erhöhtes Risiko an Magenkrebs zu erkranken mit sich. Allerdings wird angenommen, dass eine Infektion mit *H. pylori* nicht ausschliesslich negative Konsequenzen für seinen Träger hat, sondern auch gegen Allergien, chronische Entzündungen und Autoimmunerkrankungen schützen kann. Es wird vermutet, dass dieser Schutz durch die immunomodulatorischen Eigenschaften des Bakteriums hervorgerufen wird.

Auf Grund von Expressionsanalysen von CD80/CD86 Oberflächenrezeptoren und der Sekretion des Zytokines IL-12, zeigen wir nun, dass *H. pylori* in der Lage ist die LPS-induzierte Maturierung dendritischer Zellen zu inhibieren. Diese Inhibition führt zu einem halb-maturierten, tolerogenen Phänotyp und die betroffenen dendritischen Zellen sind einerseits nicht mehr in der Lage effiziente Effektor T-Zell Antworten zu vermitteln, andererseits induzieren sie die Expression des Transkriptionsfaktor FoxP3 in naiven T Zellen. Die experimentelle Depletierung dendritischer Zellen bricht die pathogen-spezifische Toleranz des Wirtes und führt zu einer besseren Kontrolle der Infektion, allerdings auf Kosten einer erhöhten Immunopathologie im Magen. Zusätzlich konnten wir in der Magenmucosa von humanen Trägern phänotypisch ähnliche, sprich dendritische Zellen, die nur wenig CD80 und CD86 exprimieren, nachweisen. Mechanistisch konnten wir zeigen, dass die Sekretion von IL-18 durch diese tolerogenen dendritischen Zellen benötigt wird, um in naiven T Zellen die Expression von FoxP3 zu aktivieren. Des Weiteren konnten wir die Notwendigkeit zweier bakterieller Faktoren, der γ-Glutamyl Transpeptidase (GGT) und des vakuolierenden Zytotoxins (VacA), für die Tolerisierung dendritischer Zellen nachweisen. Mutante Bakterienstämme, die kein GGT oder VacA produzieren, scheitern in der Tolerisierung dendritischer Zellen und als Konsequenz können diese keine regulatorischen T Zellen mehr induzieren. Beide Mutanten zeigen deutlich niedrigere Kolonnisierung im Magen eines Wirtes im Vergleich zu dem entsprechenden Wildtyp Stamm. Diese Reduzierung wird von stärkeren Th1 und Th17 Immunantworten und/oder erhöhter Magenpathologie begleitet.

Wir konnten auch zeigen, dass zwar regulatorische T Zellen von infizierten Wildtyp Mäusen, jedoch nicht regulatorische T Zellen von *Il-18*$^{-/-}$ oder *Il-18r*$^{-/-}$ Tieren, die

klinische und histopathologische Manifestation von Asthma verhindern. Ein ähnlicher Verlust des Schutzes vor Asthma ist in Mäusen zu beobachten, die mit der Δ*vacA* Mutante infiziert wurden. Dies unterstreicht zusätzlich die Wichtigkeit von VacA bei der Induktion von *H. pylori*-spezifischer Immuntoleranz.

Zusammenfassend deuten unsere Resultate darauf hin, dass die tolerogene Programmierung von dendritischen Zellen die Persistenz von *H. pylori* und den Asthmaschutz durch das Pathogen sichern und, dass der ganze Prozess abhängig von IL-18 und den bakteriellen Faktoren GGT und VacA ist.

III. ABBREVIATIONS

AP1	Activator protein 1
BabA	Antigen binding adhesin
cagA	Cytotoxin-associated gene A
***cag*-PAI**	Cag pathogenicity island
CD	Cluster of differentiation
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
GGT	γ-Glutymyl transpeptidase
H. pylori	*Helicobacter pylori*
HpaA	*H. pylori* adhesin A
IFN-γ	Interferon-gamma
IL-	Interleukin-
LPS	Lipopolysaccharides
MALT	Mucosa-associated lymphoid tissue
MAPK	Mitogen-activated protein kinase
MLN	Mesenteric lymph node
NF-κB	nuclear factor 'kappa-light-chain-enhancer of activated B-cells
NLR	NOD-like receptor
NOD	Nuleotide oligomerization domain
PAMP	Pathogen-associated molecular pattern
PRR	Pattern recognition receptor
SabA	Sialic acid binding adhesin
T4SS	Type IV secretion system
Th	T helper
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-alpha
Treg	T-regulatory cell
VacA	Vacuolating cytotoxin

TABLE OF CONTENT

1. INTRODUCTION

1.1.1 *HELICOBACTER PYLORI*

1.1.2 TAXONOMY

When Marshall *et al.* successfully cultured a novel gastric bacterium from the antral biopsy from patients with gastritis and gastric ulcers in 1982, they classified it as a new species in the genus *Campylobacter* as *Campylobacter pyloridis* (later changed to *Campylobacter pylori*) [1]. It was not before 1989 that an important stage in the development of the taxonomy of gastric microorganisms took place by creating a new genus called *Helicobacter* thereby transferring *Campylobacter pylori* to that genus [2, 3].

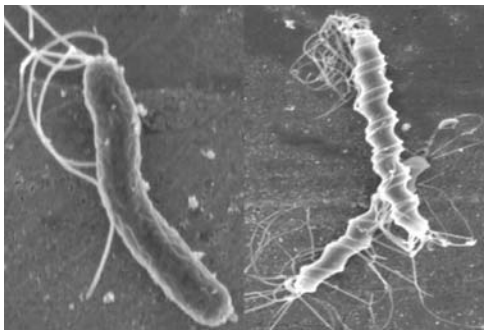


Fig.1: Different morphology of *Helicobacter spp*. S-shaped *H. pylori* with sheathed polar flagella (left) and spiral shaped *H. felis* with bipolar sheathed flagella (right). Field emission SEM. Courtesy of Lucy Thompson, University of New South Wales.

As mentioned above the first representative of the *Helicobacter* genus to be discovered was *Helicobacter pylori* (*H. pylori*), named after its primary biological niche, the pylorus of the human stomach. Subsequently, over 32 different species of *Helicobacter* have been isolated from several organisms, including cats, dogs, sheep, rodents, pigs, birds, cheethas, poultry and monkey [4]. *Helicobacter spp.* share some common features. They are gram-negative, microaerophilic bacteria possessing 4 up to 20 flagella, which make them highly motile, and they express high levels of urease, an enzyme which converts urea into carbonic acid and ammonia. They are s-shaped or curved (Fig. 1), and they have a G+C content of chromosomal DNA of 35-44 mol%. A distinctive attribute of *Helicobacter spp.* within the genus is their primary location in the host. Besides the

gastrointestinal tract, *Helicobacter spp.* have been isolated from the liver and recently from the pancreas, which was considered to be a sterile organ so far [5, 6].

1.1.2 VIRULENCE FACTORS

Bacterial virulence factors can be defined as traits or molecules that are necessary for pathogenesis, or at least, whose loss significantly reduces pathogenesis. Multiple of such factors have been proposed to contribute to *H. pylori* virulence. Among these are bacterial urease activity [7], falgellar motility [8], exotoxins [9], adhesion molecules [10], and others [11].

All *Helicobacter spp.* exhibit modest to marked urease activity *in vitro* and *in vivo* thereby enabling the bacteria to colonize the acidic environment of the stomach by providing an alkaline microenvironment [12]. Several studies support this assumption by showing that isogenic urease-negative strains are not able to colonize the stomach of gnotobiotic piglets, ferrets, and nude mice. Additionally, in contrast to their wild-type counterparts which induce gastritis after successfully colonizing their host stomach, urease-negative *Helicobacter* do not induce any signs of disease [13, 14].

H. pylori is highly motile by means of flagellar propulsion. This motility is considered as an important virulence factor, enabling *H. pylori* to travel through the extreme acidic gastric lumen to reach the more neutral environment of the mucus layer [8]. There is evidence that the degree of motility of *H. pylori* strains correlates with the degree of infectivity. Eaton *et al*. showed that the most motile strain was the most successful in terms of infection rate, while the least motile displayed only a weak rate of infection in the well-established gnotobiotic piglet model [15, 16]. In the same model, *H. pylori* strains defective in the genes coding for the minor and major flagellar proteins, FlaB and FlaA respectively, and thus impaired in their motility, colonized the host but only persisted for 2 days. In contrast, the wild-type strain colonized all piglets and persisted for at least ten days after inoculation [17], highlighting the importance of motility for the pathogen to successfully settle in the stomach of its host.

Besides traits that enable *H. pylori* to adapt to the acidic environment of the gastric tract and to reach its main biological niche, the mucus layer, there are other factors contributing to the virulence of *H. pylori*. The most prominent among them is the Cytotoxin-associated gene A (CagA) protein. The *cagA* locus is always associated with a

40-kilobase fragment of DNA known as the *cag* pathogenicity island (*cag*-PAI) [18]. The cagA protein is actively translocated from the bacterium to the host cells by a type IV secretion system (T4SS). Upon translocation, CagA gets phosphorylated inside the host cell [19, 20]. Strikingly, biopsies from patients suffering from severe gastric diseases ranging from chronic active gastritis to mucosa-associated lymphoid tissue (MALT) lymphoma or gastric adenocarcinoma harbor the *cagA* gene in approximately 90% of all the cases, establishing a direct correlation of the presence of the *cagA* gene with disease [18]. The effector functions of CagA are manifold. Morphological changes in epithelial cells by actin polymerization [21], promotion of inflammation [22-24], dysregulation of proliferation [25], and attenuation of T-cell apoptosis have been attributed to CagA translocation [26].

Another wide-spread virulence factor among *Helicobacter* species is the exotoxin vacuolating cytotoxin VacA. This multifunctional cytotoxin intoxicates a variety of cell types, including epithelial cells and immune cells, resulting in an array of cellular responses. Among them we find induction of vacuolation [9], alteration in mitochondrial membrane permeability [27], inhibition of antigen presentation on B-lymphocytes [28], and inhibition of T-cell proliferation [29]. Besides the effects on T- and B-lymphocytes described above, which might result in localized immunosuppression, VacA additionally induces pro-inflammatory effects by stimulating tumor necrosis factor- (TNF-)α- and interleukin- (IL-)6-secretion in mast cells [30]. Thus, VacA earns its name as a multifunctional toxin by being both an immunosuppressor and an immunostimulant.

Many *H. pylori* adhesins have been implicated to promote the bacterium`s virulence by enabling its adherence to gastric epithelial cells, thereby enhancing the activity of other bacterial molecules involved in virulence. This outer membrane family of proteins includes the blood group antigen binding adhesin (BabA), the sialic acid binding adhesin (SabA), which bind to Lewis b and sialyl Lewis x receptors, respectively, and the *H. pylori* adhesin A (HpaA) [31-34].

γ-Glutamyl transpeptidase (GGT) has recently been added to the list of virulence factors associated with *H. pylori*. Kim *et al.* showed that GGT induces cell-cycle arrest at the G1 to S phase transition in human T-cells [35]. In addition, recombinant GGT leads to the generation of H_2O_2 in primary gastric epithelial and AGS cells, resulting in the activation of the NF-κB pathway and up-regulation of IL-8 [36]. Most strikingly, *H. pylori* strains recovered from patients with peptic ulcer disease showed a significantly higher GGT activity than strains isolated from patients suffering from nonulcer dyspepsia [35, 36]. The

high number of involved virulence factors, and the antigenic variation of the respective genes in different *H. pylori* strains, underlines the tremendous capacity of the bacteria to continuously adapt to their hostile environment [37].

1.1.3 PREVALENCE AND TRANSMISSION

H. pylori is one of the most widespread bacterial infections, affecting approximately 50% of the world`s population [38]. The prevalence of *H. pylori* infection varies widely by geographic area, socioeconomic status, age, and ethnicity. Infection rates are higher in developing (≥ 70%) than in developed countries (≤ 40%) and seem to be inversely correlated with hygiene standards [39](Fig. 2). Most infections are acqired during early childhood with a lifelong persistence if not treated [40, 41]. Spontaneous clearance can occur, but is rare (0.2-1%/year). Humans appear to be the only reservoir of *H. pylori*, although several studies show a possible correlation of domestic animals (cats and sheep) harbouring the bacteria with a higher frequency of infection in the owners. Starting from humans as the reservoir, the most likely route of transmission is from person to person, by either the fecal-oral route or by the oral-oral route. Additionally, transmission via contaminated food or water is regarded as possible, although not convincingly proven [39].

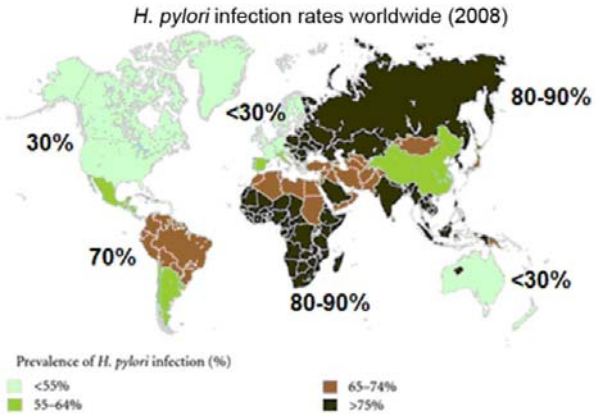


Fig. 2: Prevalence of *H. pylori* in asymptomatic adults. Data are from population-based surveys of healthy adults. Adapted from [42].

1.2 *HELICOBACTER PYLORI* AND GASTRIC CANCER

1.2.1 EPIDEMIOLOGY

Besides the induction of gastritis, gastric ulcers, and duodenal ulcers, *H. pylori* infection can lead to the development of gastric adenocarcinomas and MALT lymphoma. Therefore, *H. pylori* was classified as a class I carcinogen by the World Health Organization in 1994 [43]. Although the majority of infected individuals remain asymptomatic, despite a lifelong persistence of the infection, 1-2% of the carriers will eventually develop gastric adenocarcinoma [44] or MALT lymphoma (below 1%) [45]. Although gastric cancer-related deaths are constantly decreasing since 1930 in industrialized countries, it is estimated that approximately 738`000 deaths occurred in 2008, making this cancer one of the top two killers from cancer worldwide. One major determinant for the favorable negative trend is the reduction in chronic *H. pylori* infections in most parts of the world [46]. Although a negative trend has to be appreciated, *H. pylori* infection-related diseases are still a considerable burden for the public health system.

1.2.2 MECHANISMS OF CARCINOGENESIS

In general, development of gastric cancer is a multistep process initially starting by diffuse infiltration of the gastric mucosa by lymphocytes after acquiring the infection. This so-called chronic active nonatrophic gastritis advances slowly to the multifocal atrophic type of gastritis, which is characterized by loss of glands (atrophy) at the antrum-corpus junction, followed by intestinal metaplasia. At this precancerous stage, the glands and the foveolar epithelium are replaced by "intestinal-like" cells. Subsequently, dilatation and branching of gastric glands and atypical morphology and sustained mitotic activity of epithelial cells can be observed. After acquiring this dysplastic stage, most of the affected patients will progress to early carcinomas and advanced, invasive adenocarcinoma, suggesting that genetic alterations have already taken place [47, 48](Fig. 3). Gastric adenocarcinoma constitutes over 85% of all gastric malignancies. However, *H. pylori* can additionally cause MALT lymphoma of the stomach, which constitutes

roughly 3% of all gastric malignancies and 10% of all non-Hodgkin lymphomas [49, 50]. Clinical, epidemiological, and experimental data have provided strong evidence for a causal involvement of *H. pylori* in the etiology of this malignancy [45].

Although roughly 50% of the world population is infected with *H. pylori*, only a minority will develop gastric cancer, highlighting the importance of the combination of bacterial factors, environmental traits, and the host immune response in the progression toward malignant disease. Different virulence factors have been associated with a higher risk of gastric cancer. $CagA^+$ strains in particular are more cancerogenic in humans and in animal models of disease [51, 52]. Besides bacterial factors, the host immune response plays an important role in the development of malignant lesions. Polymorphisms in the genes for IL-1β, the IL-1β receptor, and IL-10 correlate with an increased risk of gastric cancer [52-54]. Additionally, *H. pylori* infection leads to the induction of high levels of reactive oxygen species and enhanced epithelial cell proliferation in the gastric tissue [55]. More recently Toller *et al.* showed the induction of double strand breaks in epithelial

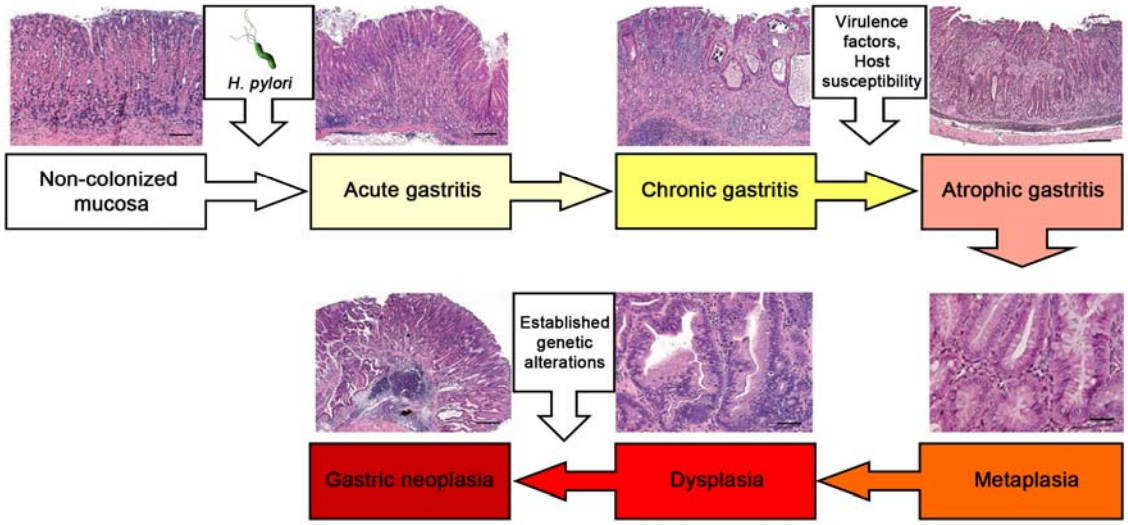


Fig. 3: Histological progression of *Helicobacter*-induced gastric cancer in a B6129 mouse model. Adapted from (56, 57)

cells upon *H. pylori* exposure [58]. In theory, these effects of *H. pylori* on the gastric tissue enhance the risk of acquiring genetic alterations and thus driving the development of cancerous lesions. The induction of genetic alterations by the pathogen suggests that

there is a "point of no return", and that disease progression in later stages is not exclusively depending on a persistent infection of the host with the microorganism [59]. Support for this hypothesis comes from follow-up studies after eradication therapy of the bacteria. Regression has been observed upon eradication for acute, chronic and atrophic gastritis, but not for metaplastic and dysplastic lesions, suggesting that that persistent infection is not required for proceeding through the late disease stages of carcinogenesis [60, 61].

1.2.3 *HELICOBACTER PYLORI*: FRIEND OR FOE?

As discussed in the previous section, persistent infection with *H. pylori* causes gastritis and predisposes carriers to a high gastric cancer risk. Besides its pathological effects in the stomach, it is speculated that *H. pylori* is causally linked to a variety of extragastric diseases, some of which include functional dyspepsia [62], gastroesophageal reflux disease [63], cardiovascular diseases [64-66], iron deficiency anemia [67], idiopathic thrombotic purpura [68], and others [69].

Considering the multiple pathological effects induced by *H. pylori* in its human host, we might argue that this microorganism should be eradicated by the wide-spread use of antibiotics or development of vaccines. On the other hand, only a minority of carriers is adversely affected by the infection and an indiscriminate use of antibiotics may lead to bacterial resistance. More importantly, there is growing evidence that *H. pylori* may prevent against asthma [70-73], allergies and other chronic inflammatory diseases [74]. This inverse correlation is most evident in Western societies that have largely eliminated *H. pylori* (Fig. 2 and 4A) infections due to frequent use of antibiotics in childhood and better sanitary conditions. In these populations, the rates of such diseases have reached epidemic proportion, putting a huge burden on the public health system [75](Fig. 4B). Therefore any decision on complete eradication of *H. pylori* might be premature and even detrimental, especially since Arnold *et al.* showed recently that neonatal infection with *H. pylori* protects mice against the clinical and histopathological symptoms of asthma [70]. Understanding the mechanisms of *H. pylori*-mediated asthma protection would have a great impact on the decisions how to deal with this pathogen.

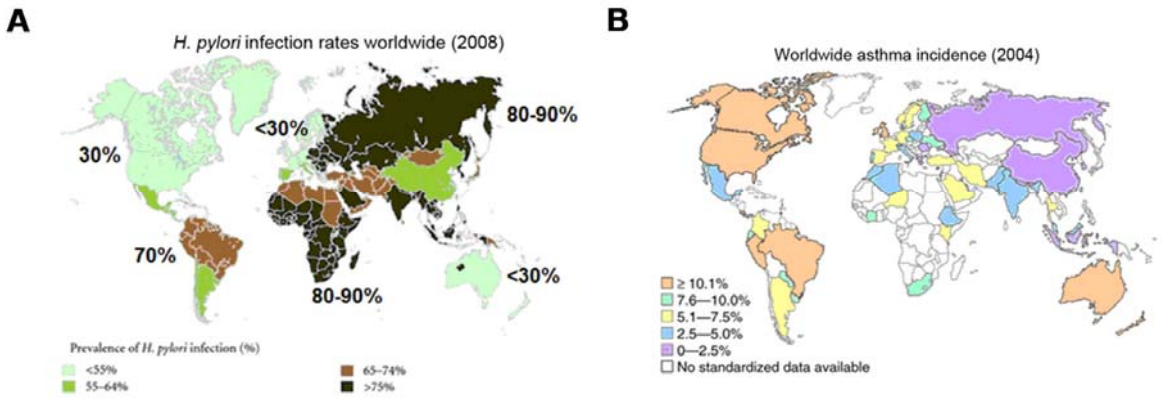


Fig. 4: The decline of H. pylori infection rates inversely correlates with an increase in asthma incidence. Prevalence of *H. pylori* in asymptomatic adults. Data are from population-based surveys of healthy adults (**A**). Worldwide asthma incidence (**B**). Figure adapted from [42, 76].

1.3 IMMUNE RESPONSES TO *HELICOBACTER PYLORI*

1.3.1 INNATE IMMUNITY

The innate immune response in the stomach is initiated upon binding of pathogen- or damage-associated molecular patterns (PAMPs/DAMPs) or to pattern recognition receptors (PRR) located at the cell surface, endosomal membranes and the cytosol. These receptors recognize several PAMPs such as flagellin, peptidoglycan, and lipopolysaccharide (LPS). Detailed studies on the role of various PRRs in the context of a *H. pylori* infection have been performed. Namely, the involvement of Toll-like receptors (TLRs), cytosolic nucleotide oligomerization domain (NOD)-like receptors (NLRs), C-type lectins and caspase-recruiting domain proteases have been under investigation. TLRs and NLRs are expressed by the first component of the innate immune system to be encountered by *H. pylori*, the epithelial cell lining of the gastric mucosa. Recognition of *H. pylori* by TLR-2 [77, 78] and NOD-1 [24, 79] results in the activation of MAPKs and the transcription factors NF-κB and AP-1 [24, 79] followed by subsequent secretion of various pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α, IL-8 and chemokines such as MCP-1, GRO-α, MIP-1α, and IL-8 in a T4SS-dependent manner [80-82]. Whether the

classical receptor for bacterial LPS, TLR-4 [78, 83, 84], and/or the classical receptor for bacterial flagellin TLR-5 [77, 78, 83-85] are required for this response by the gastric epithelium is still controversial. The local release of cytokines and chemokines by the epithelial cells of the gastric mucosa leads to the recruitment of various types of immune cells, including granulocytes, monocytes and lymphocytes. Neutrophils, a characteristic infiltrating cell type in *H. pylori* infections, upregulate IL-12, IL-23 and TNF-α via engagement of TLR-2 and subsequent NF-κB activation upon contact with the pathogen [86]. Similarly, *H. pylori* activates TLR-2 and TLR-4 on macrophages leading to the secretion of IL-12 and IL-10 or L-6 and IL-1β, respectively [87]. Interestingly, B-cells exposed to *H. pylori* extract produce large amounts of the regulatory cytokine IL10 in a TLR-2-/MyD-88-dependent manner. Additionally, they acquire the ability to efficiently induce IL-10 expression in co-cultured, naive $CD4^+$ T cells, thereby converting the T cells to T-regulatory-1 (Tr1)-like cells with suppressive activity [88]. Several other studies focused on the role of dendritic cells (DCs) in the context of a *H. pylori* infection, since they represent a critical link between the innate and the adaptive immune response. DCs can penetrate the gastrointestinal epithelial barrier *in vivo* and both luminal and subepithelial interactions of *H. pylori* or bacterial products with DCs have been reported [89-91]. However, data on the activation of DCs by *H. pylori* are controversial. Pulsing of human peripheral blood monocyte-derived DCs (PMDCs) with *H. pylori ex vivo* leads to the generation of IL-12 and simultaneously IL-10 with subsequent induction of T helper (Th) 1 or Th2/regulatory T-cell (Treg) responses, respectively [92, 93]. Yet another study co-cultured pulsed PMDCs with naive T-cells resulting in TNF-α, interferon-(IFN-) γ, and IL-2 secretion, indicative of a T-cell differentiation towards a Th1 phenotype [94] (Fig. 5). Geijtenbeek *et al.* showed that *H. pylori*-specific carbohydrate variants can bind to the C-type lectin DC-specific ICAM-3-grabbing nonintegrin (DC-SIGN/CD209) on human DCs resulting in enhanced expression of IL-10 but downregulation of IL-12 and IL-6 secretion, thus suppressing Th1 polarization in favour of persistence of the bacteria in the host [95, 96]. The involvement of *H. pylori*-activated TLR signalling on human PMDC is not well characterized due to methodological limitations. It is believed that TLR-4 as well as TLR-2 mediates the observed DC activation and cytokine secretion [95]. More data are available on the interaction of *H. pylori* with PRRs on murine DCs. Murine bone marrow-derived DCs (BMDCs) phagocytose *H. pylori* resulting in the expression of pro-inflammatory cytokines IL-1α, IL-1β, IL-6 and the anti-inflammatory cytokine IL-10 in a TLR-2-, TLR4-, and TLR9-dependent manner [97]. However, only modest secretion of IL-

12 is observed, resulting in diminished activation of splenocyte proliferation and INF-γ secretion compared with that induced by another pathogen that induces gastritis in mice,

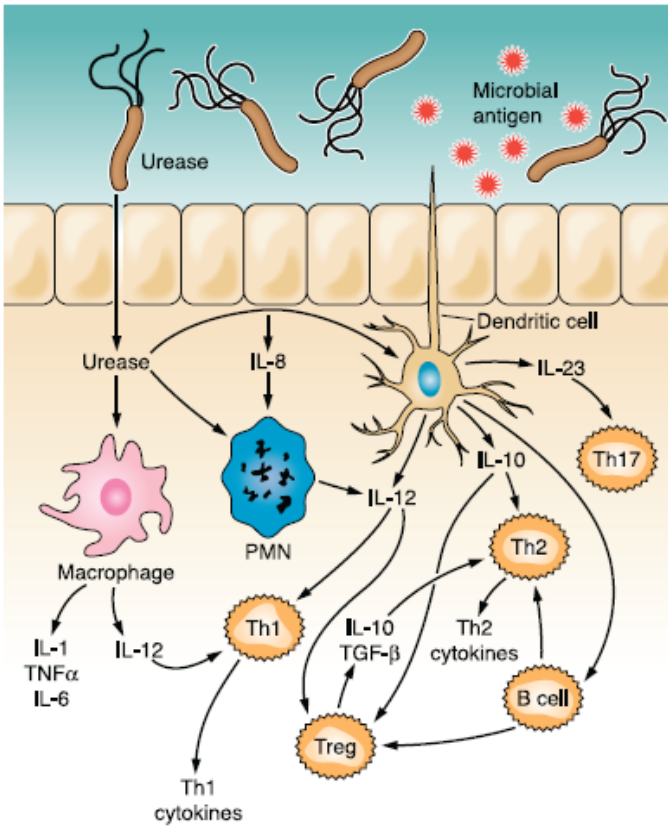


Fig. 5: DCs are the critical link between innate and adaptive immune responses against *H. pylori* in the gastric mucosa. DCs penetrate the gastric epithelial lining and sample *H. pylori* antigens in the lumen. Activation of DCs by *H. pylori* antigens lead to the secretion of different cytokines, including IL-12, IL-23 and IL-10 and the induction of either Th1, Th2, Treg, or Th17 responses. The adaptive T cell responses are further amplified by the recruitment of polymorphonuclear cells and macrophages to the site of infection. From [56].

Acinetobacter iwoffi. Additionally, the release of IL-12 upon stimulation of BMDC with *Acinetobacter iwoffi* is inhibited in the presence of *H. pylori* secreted factors [98]. Recently, it has been shown that *H. pylori*-pulsed BMDCs enhance the production of IL-23, promoting the proliferation of Th17 cells, a pathway that has been implicated, amongst others, in colitis, a disease sharing some mechanistical features with *H. pylori* induced gastritis [99, 100]. Besides TLRs, the intracellular retinoic acid-inducible gene I (RIG-I) receptor interacts with *H. pylori* RNA resulting in the induction of type 1 interferons. This response was independent of the known TLR adaptor proteins Myd88 and TRIF [97].

A third component of the DC activation program is composed of the NLRs NALP3 and NLRC4. Both of these intracellular signaling complexes are integral components of inflammasomes recognizing different PAMPs and DAMPs, including a variety of gram-positive and gram-negative bacteria. This recognition leads to the activation of the cysteine protease caspase-1 and the subsequent proteolytical cleavage of pro-IL-1β and

pro-IL-18 into their mature, bio-active forms [101]. Although stomach-specific overexpression of the pro-inflammatory cytokine IL-1β in mice leads to spontaneous gastric inflammation and cancer [54], the role of NALP3- and NLRC4-inflammasomes in the context of a *H. pylori* infection has not been addressed in detail yet. Further studies on the possible role of the highly dynamic NLR(s)-caspase-1-inflammasome(s) complex(es) in different cell types including epithelial cells, granulocytes and monocytes are needed.

Taken together, much effort has been made to elucidate the role of innate immunity in *H. pylori* infections *in vitro* and *in vivo,* however, no clear trend can be established on whether the pathogen fully activates or evades the various components of the innate immune system.

1.3.2 ADAPTIVE IMMUNITY

Initially, it was believed that B-cell mediated humoral responses against *H. pylori* are decisive in the induction of protective immunity against the bacteria. Although *H. pylori* elicits antibody responses in the mucosa and the serum, it quickly became clear that the humoral arm of the immune system plays only a minor role in fighting the pathogen [102]. To date, T cells are considered to be the main orchestrators of *H. pylori*-mediated adaptive immunity [102-104]. Infiltrating T cells into the antrum of infected hosts mainly consist of $CD4^+$ and $CD8^+$ T cell subsets in mice and humans. Although both subsets show similar cytokine profiles, namely IFN-γ and TNF-α, upon activation by *H. pylori*-specific antigens complexed to MHC class II molecules [105], no crucial role for $CD8^+$ T cells has been shown, except in the absence of $CD4^+$ T cells [106-108]. The abundance of IFN-γ and TNF-α in the gastric mucosa of infected mice and humans clearly indicates a Th1 polarization, and especially IFN-γ expression is necessary to drive *H. pylori*-induced gastritis and pathology [104, 109].

In contrast, a subset of individuals develops either a Th2 or a mixed Th1/Th2 profile in response to the infection. The secretion of IL-4 or both, IFN-γ and IL-4, respectively, has been suggested to protect from severe gastric pathology [110]. It is generally believed that the Th1 cell subset protects the host against intracellular bacteria and the Th2 cell subset is crucial for immunity to parasites, the recently described Th17 cells are involved in the clearance of extracellular bacteria and thus very interesting in the context of a *H.*

pylori infection [111]. Indeed, IL-17 secretion from tissue resident T cells, triggered by the release of IL-23 by DCs and macrophages, is detected at early and late stages of *H. pylori* infection [98, 112-114]. The degree of inflammation and neutrophil infiltration is lower in IL-17 knock-out mice or upon IL-17 neutralization. Although the lack of IL-17 leads to decreased inflammation, the bacterial burden is higher in wild type mice as compared to knock-out mice, suggesting that IL-17 prevents inflammation-driven pathology but does not contribute to bacterial clearance [115, 116]. Interestingly, Th17 and Th1 responses are suppressed in *H. pylori*-infected mice by a developmentally related population of $CD4^+$ T-cells, namely peripheral induced $FoxP3^+$ Tregs [117]. The suppression of T-effector responses by Tregs in mice leads to the reduction of the degree of gastritis and to higher bacterial colonization levels, whereas depletion of Tregs reduced the bacterial burden and induced more severe gastritis and preneoplastic changes [118, 119]. The key role of Tregs in the context of *H. pylori* infections is especially evident in the pediatric cohort. $FoxP3^+$ cell numbers are higher, but IL-17 and IL-23 levels are lower in the gastric mucosa of infected children than in the mucosa of infected adults [120, 121]. Additionally, Arnold *et al*. demonstrated that neonatally infected mice are highly colonized and protected against any signs of inflammation, gastritis or pre-cancerous changes. This neonatal tolerance is mediated by peripherally induced Tregs, which efficiently suppress Th1 and Th17 responses [122]. The protective effect is lost upon depletion of $CD25^{high}$ Tregs [99, 123, 124]. Moreover, in asymptomatic carriers of *H. pylori*, IL-10-expressing Tregs are particularly abundant in the mucosa compared with patients suffering from peptic ulcer disease [123, 125]. High abundance of IL-10-expressing Tregs is directly correlated to high colonization scores and vice versa [126]. This induction of Tregs by *H. pylori*, together with its weak immunogenicity, probably enables the bacteria to evade immune surveillance by dampening local adaptive immune responses. The mechanism of induction of such Tregs is not entirely clear. Better insights into this mechanism are crucial to overcome the tremendous obstacles encountered in the development of a potent, sterilizing vaccine.

1.4 DENDRITIC CELLS AND IMMUNE TOLERANCE

As disscussed in section 1.3.2 peripheral induced $FoxP3^+$ Tregs promote persistent infection with *H. pylori* and prevent excessive immunopathology in the gastric epithelium

of the host [119, 122]. In contrast to naturally occuring Tregs, which originate in the thymus, inducible Tregs are induced in peripheral tissues upon antigenic stimulation by DCs [127-130]. The cruical role of DCs in orchestrating innate and adaptive immunity is commonly recognized by workers in the field. DCs are a specialiced leukocyte cell type, that aquires, processes and present antigens to T cells. DCs have first been described as potent stimulators of adaptive immunity, but there is mounting evidence that these specialized antigen-presenting cells can additionally establish and maintain immunological tolerance [131].

As mentioned above, tissue resident immature DCs constantly sample their environment for PAMPs and DAMPs. Upon sensing of such microbial and tissue damage signals via TLRs, NLRs, C-type lectins and others [132-134], DCs upregulate a variety of membrane receptors involved in antigen presentation and co-stimulation such as MHC-II, CD40, CD80, and CD86 as well as different cytokines that promote and modulate T cell polarization, such as IL-12, IL-6, IL-1β, IL-8 and IL-2 [92]. Additionally, members of the NF-κB family are activated upon recognition of such PAMPs and DAMPs [135]. Simultanously, DCs loose their capacity to sample antigens upon maturation but gain the capacity to migrate to the draining lymph nodes by the upregulation of homing receptors such as C-C chemokine receptor type 7 (CCR7) [92, 136]. Now fully mature, DCs present three signals to circulating lymphocytes. Signal one is the presentation of the antigenic stimulus via MHC-II, signal two is provided by costimulatory molecules and signal three is provided by DC-derived cytokines [92, 137]. The presentation of antigen with concomitant co-stimulation and cytokine secretion is needed to induce full-fledged effector T cells in the draining lymph nodes [134, 138, 139](Fig. 6A). Whereas fully mature DCs possess primarily immunogenic functions, various phenotypes of tolerance-promoting DCs have been described. In particular, thymic plasmacytoid DCs promote the induction of natural $FoxP3^+$ Tregs in the thymus to induce and maintain central suppressive tolerance [127, 140]. The signals provided by the thymic environment to render plamacytoid DCs tolerogenic are not completely understood, but probably involves thymic stromal lymphoietin secreted by Hassall`s corpuscles in the thymic medulla [141-143].

Peripheral suppressive tolerance is mainly mediated by DCs within the mucosa and the lamina propria of different organs (e.g. lung, intestine, skin). For instance, intestinal DCs sample the lumen of the intestinal tract and migrate to the draining lymph nodes upon antigen uptake, where they induce FoxP3-expressing Tregs [144, 145]. This tolerogenic capacity of intestinal DCs is belived to be induced by the mucosal environment of the

intestine, which is rich in TGF-β, retinoic acid, IL-10 and other anti-inflammatory, tolerance-promoting factors [146-149]. Intestinal DCs believed to possess the most potent Treg inductive capacity express CD103, an integrin which is regulated by TGF-β signaling [150, 151].

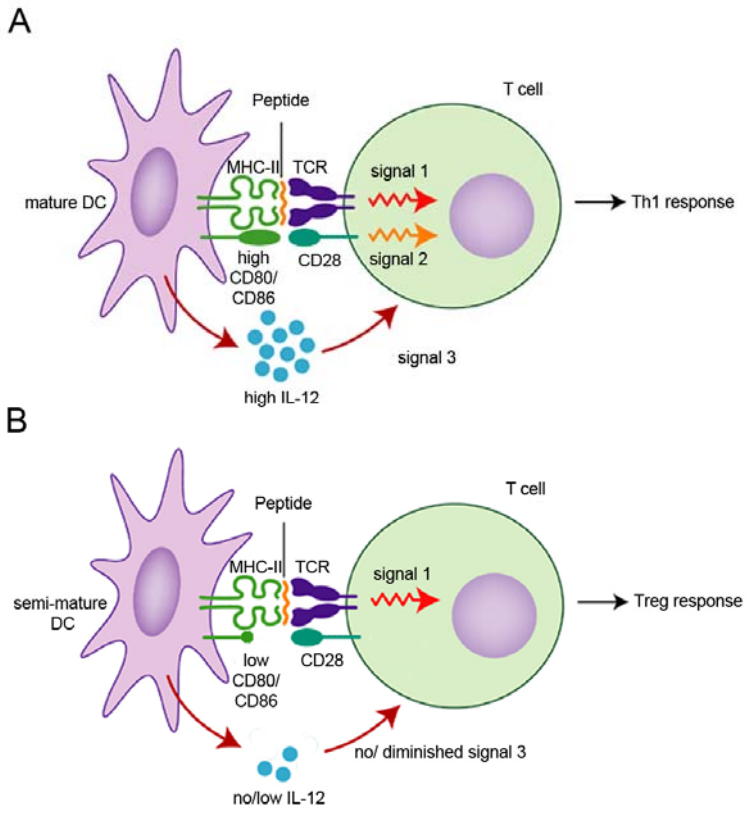


Fig. 6: The maturation status of DCs influences T cell polarization: Presentation of an antigenic stimulus with concomitant co-stimulation via CD80/CD86 and secretion of IL-12 leads to Th1 polarization of naive T cells (**A**). Presentation of an antigen via MHC-II without concomitant co-stimulation and low secretion of pro-inflammatory cytokines preferentially induces FoxP3-expressing Tregs (**B**). Adapted from [152].

Another key factor for the induction of Tregs is an environment containing little or no pro-inflammatory cytokines, like IL-12 and IL-6, or co-stimulatory molecules such as CD40, CD80, and CD86 in combination with an antigenic stimulus [153]. These circumstances are provided by so-called semi-mature DCs. Semi-mature DCs provide an antigenic stimulus by MHC-II presentation in the absence of co-stimulation and cytokine secretion to naive T cells, thereby promoting Treg differentiation [154-157] (Fig 6B).

Besides factors released by the local surroundings of tissue-resident DCs, a variety of other inputs have been implicated in the induction of tolerogenic DCs. Among them we find certain pathogens, which have evolved mechanisms that exploit Tregs to evade immune surveillance [158-160]. For instance, helminths release factors that mimic immunosuppressive molecules like TGF-β, thereby promoting the induction of tolerogenic, Treg-inducing DCs [159]. Many other microbial or viral factors and toxins have been shown to act similarly by prompting DCs to secrete anti-inflammatory cytokines, like IL-10 or TGF-β [161-164]. Last but not least, Tregs, once established, have the capacity to render DCs tolerogenic by producing TGF-β and IL-10 [165]. This mutual influence on each other leads to the induction of more Tregs, a mechanism sometimes referred to as "infectious tolerance".

In summary, the instruction of DCs to become tolerogenic seems to be a result of a crosstalk between DCs and their local environment, already established Tregs and/or certain pathogens, mediated by different tolerance-promoting factors such as IL-10, TGF-β, retinoic acid and/or bacterial toxins.

2. AIMS OF THE STUDY

2.1 ELUCIDATING THE ROLE OF DENDRITIC CELLS IN *HELICOBACTER PYLORI* INFECTIONS

DCs are considered as the critical link between innate and adaptive immunity. However, little effort has been made to elucidate the role of these professional antigen presenting cells in the context of a *H. pylori* infection *in vivo*. As discussed in section 1.3.1, the data gathered on the role of DCs are rather unclear. Some reports state that DCs primed with *H. pylori* induce fully-fletched Th1 polarized T cells, other results point into another direction by showing that *H. pylori*-experienced DCs induce Th2 polarized subsets of T cells. Additionally, some studies suggest that DCs can secrete IL-23 upon phagocytosis of the bacteria, subsequently inducing Th17 polarized T cells. Moreover, it is possible that the abundant Treg subset in the gastric mucosa of infected mice and humans can be induced by DCs.

Surprisingly, most of the work was done *in vitro* using either human PMDCs or murine BMDCs, although mouse models and *H. pylori* strains, recapitulating the multistep process of cancer development, colonization behavior and host immune responses are available. Our group described the use of the *H. pylori* strain pre-mouse Sydney strain 1 (PMSS1) to infect C57BL/6 mice as a suitable model to study *H. pylori*-host interactions and disease progression [122]. Since PMSS1 readily colonizes C57BL/6 mice, a variety of DC-related knock-out strains in a C57BL/6 background are available for *in vivo* studies.

Therefore we assessed the role of DCs in *H. pylori* PMSS1 infections using various mouse knock-out strains or the depletion of DCs *in vivo*, in addition to various *in vitro* assays, utilizing BMDCs. Additionally, we were interested in bacterial factors affecting DC activation and function. Therefore we assessed the impact of different *H. pylori* strains on DCs as compared to their parental, wild-typ strains.

3. RESULTS

The results gathered during my PhD thesis are summarized by the following published articles.

In the first article we show that *H. pylori* re-programs DCs toward a tolerogenic phenotype. *H. pylori*-experienced DCs efficiently induced $CD25^+FoxP3^+$ Tregs, while failing to induce robust T cell effector functions. Partial depletion of DCs in neonatal infected mice was sufficient to break *H. pylori*-specific tolerance and resulted in improved control of the infection, which was accompanied by increased T cell-driven immunopathology. The ability of *H. pylori*-experienced DCs to induce Tregs was dependent on DC-derived IL-18. Additionally, the protective effect of $CD25^+FoxP3^+$ Tregs from infected wild-type mice in an experimental model of asthma was lost if we used $CD25^+FoxP3^+$ Tregs from either *IL-18*$^{-/-}$ or *IL-18r1*$^{-/-}$ mice.

In the second paper we highlight that the depletion of DCs during the challenge phase with *H. pylori* significantly improves vaccine-induced protective immunity. We link this observation to the capacity of *H. pylori*-experienced mesenteric lymph node (MLN) DCs to induce Tregs *in vivo*. The main purpose of the paper is to introduce the mycobacterial adjuvant CAF01 as possible candidate for human *H. pylori* vaccination.

The third publication reviews how *H. pylori* manipulates innate and adaptive immunity of the host in order to establish persistent infection. We discuss several key components and signaling pathways of the immune system, which are preferentially targeted by the pathogen to evade immune surveillance and to reprogram it towards tolerance rather than immunity.

Three publications are listed in the appendix since they either do not fit the scope of this thesis or are not yet published (but submitted). The first, submitted publication deals with

bacterial factors involved in tolerogenic re-programming of DCs and the consequences for persistent infection and immunopathology. Although placed in the appendix, most of the results are discussed in section 4.

Two further publications to which I either share the first-authorship with Daniela B. Engler or to which I contributed are additionally presented in the appendix. They are not presented nor discussed in the result or discussion part, respectively, since both publications are outside of the main scope of this thesis. The first article addresses the role of microRNA-155 in the T cell-mediated control of *H. pylori* infections and the induction of colitis. The second publication deals with the T cell antigenicity of the *H. pylori* virulence factor CagA.

3.1 DC-DERIVED IL-18 DRIVES TREG DIFFERENTIATION, MURINE *HELICOBACTER PYLORI*-SPECIFIC IMMUNE TOLERANCE, AND ASTHMA PROTECTION

Article published in Journal of Clinical Investigation, 2012

Authors: Mathias Oertli, Malin Sundquist, Iris Hitzler, Daniela B. Engler, Isabelle Arnold, Sebastian Reuter, Joachim Maxeiner, Malin Hansson, Christian Taube, Marianne Quiding-Järbrink, and Anne Müller

Contributions: I planned, conducted and analyzed the data summarized in figure 1-3, 5, 7-8, and parts of figure 4. MS, MH and MQJ planned, conducted and analyzed the data summarized in figure 6. SR, JM and CT conducted and analyzed the data summarized in figure 4 and 9. DBE planned and conducted parts of the experiment summarized in figure 9. IH provided figure S3 A and B. AM suggested and planned experiments, helped with data analysis and wrote the manuscript

Research article

DC-derived IL-18 drives Treg differentiation, murine *Helicobacter pylori*–specific immune tolerance, and asthma protection

Mathias Oertli,[1] Malin Sundquist,[2] Iris Hitzler,[1] Daniela B. Engler,[1] Isabelle C. Arnold,[1] Sebastian Reuter,[3] Joachim Maxeiner,[3] Malin Hansson,[2] Christian Taube,[3,4] Marianne Quiding-Järbrink,[2] and Anne Müller[1]

[1]Institute of Molecular Cancer Research, University of Zürich, Zürich, Switzerland. [2]Department of Microbiology and Immunology, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden. [3]III. Medical Clinic, Johannes Gutenberg University, Mainz, Germany. [4]Department of Pulmonary Medicine, Leiden University Medical Center, Leiden, The Netherlands.

Persistent colonization with the gastric bacterial pathogen *Helicobacter pylori* causes gastritis and predisposes infected individuals to gastric cancer. Conversely, it is also linked to protection from allergic, chronic inflammatory, and autoimmune diseases. We demonstrate here that *H. pylori* inhibits LPS-induced maturation of DCs and reprograms DCs toward a tolerance-promoting phenotype. Our results showed that DCs exposed to *H. pylori* in vitro or in vivo failed to induce T cell effector functions. Instead, they efficiently induced expression of the forkhead transcription factor FoxP3, the master regulator of Tregs, in naive T cells. Depletion of DCs in mice infected with *H. pylori* during the neonatal period was sufficient to break *H. pylori*–specific tolerance. DC depletion resulted in improved control of the infection but also aggravated T cell–driven immunopathology. Consistent with the mouse data, DCs infiltrating the gastric mucosa of human *H. pylori* carriers exhibited a semimature DC-SIGN$^+$HLA$^-$DR^{hi}CD80^{lo}CD86^{lo} phenotype. Mechanistically, the tolerogenic activity of *H. pylori*–experienced DCs was shown to require IL-18 in vitro and in vivo; DC-derived IL-18 acted directly on T cells to drive their conversion to Tregs. CD4$^+$CD25$^+$ Tregs from infected wild-type mice but not *Il18*$^{-/-}$ or *Il18r1*$^{-/-}$ mice prevented airway inflammation and hyperresponsiveness in an experimental model of asthma. Taken together, our results indicate that tolerogenic reprogramming of DCs ensures the persistence of *H. pylori* and protects against allergic asthma in a process that requires IL-18.

Introduction

DCs are predominantly known for their function as potent inducers of adaptive immunity. However, there is now increasing evidence that certain populations of poorly immunogenic DCs initiate and maintain immunological tolerance through induction of anergy, deletion of autoreactive T cells, and the instruction and differentiation of Tregs (1). Under steady-state conditions, tissue-resident immature DCs express low levels of MHC class II (MHCII), costimulatory molecules, and proinflammatory cytokines but may transform to fully mature DCs upon antigen uptake, accompanied by the concomitant sensing of pathogen-associated molecular patterns (PAMPs) or of "danger" signals released by tissues in distress (2). Such PAMPs or danger-associated molecular patterns (DAMPs) are detected by membrane-bound TLRs or cytoplasmic Nod-like receptors (NLRs) (3, 4) and induce the expression of MHCII, CD40, CD80, and CD86 as well as a number of proinflammatory and T cell–activating cytokines, including IL-1β, IL-6, and IL-12 (2). Immature DCs that have taken up antigen, but have not simultaneously been exposed to TLR or NLR ligands, are thought to acquire a semimature state, characterized by high levels of MHCII but low or no expression of costimulatory molecules or proinflammatory cytokines; such semimature CD11c$^+$MHCII^{hi}CD80^{lo}CD86^{lo} DCs are believed to exhibit tolerogenic (as opposed to stimulatory or immunogenic) properties (1). Tolerogenic DCs function by converting naive T cells into FoxP3$^+$ Tregs with suppressive activity; Treg induction is achieved through antigen presentation in the absence of costimulatory signals or cytokines, either alone or in combination with the production of soluble and membrane-bound tolerogenic factors, such as IL-10, TGF-β, retinoic acid, and programmed death ligands (1, 5).

Persistent infection with the gram-negative gastric bacterial pathogen *Helicobacter pylori* results in chronic gastritis (6) and predisposes carriers to a high risk of developing gastric and duodenal ulcers, gastric cancer, and gastric mucosa-associated lymphoid tissue lymphoma (7–9). We and others have shown previously that MHCII-restricted T cells are required for the control of this extracellular pathogen under conditions of experimental infection in naive mice (10) and for the development of vaccine-induced protective immunity (11, 12). Th1-polarized, pathogenic CD4$^+$ T cells further represent the driving force behind the infection-associated gastric preneoplastic immunopathology that manifests histologically as atrophic gastritis, epithelial hyperplasia, and intestinal metaplasia in infected rodents and in a subset of chronically infected humans (10, 13–15). Consequently, targeting T cells pharmacologically prevents and even reverses the gastric immunopathology associated with chronic *Helicobacter* infection (16, 17). Interestingly, the outcome of the *Helicobacter*/host interaction varies dramatically depending on the age at the time of infection. Whereas mice infected as fully immunocompetent adults develop preneoplastic lesions within 2 to 4 months of infection with virulent *Helicobacter* strains, mice infected during the neonatal period are protected against gastric immunopathology due to their development of immune tolerance to the pathogen (18).

Conflict of interest: The authors have declared that no conflict of interest exists.
Citation for this article: *J Clin Invest.* 2012;122(3):xxxx–yyyy. doi:10.1172/JCI61029.

The depletion of Tregs breaks neonatally acquired tolerance and results in a dramatic reduction of bacterial loads and the development of Th1-associated immunopathology (18). Tregs induced during neonatal infection are further both required and sufficient to mediate the *H. pylori*–induced, but antigen-independent, cross-protection against allergic asthma that is evident in human carriers of *H. pylori* (19–22) and in experimentally infected mice (23). In an ovalbumin sensitization/challenge model of allergic asthma, neonatally infected mice were completely protected against the clinical and histopathological features of asthma, i.e., airway hyperresponsiveness, tissue inflammation, and bronchoalveolar eosinophilia (23). Depletion of Tregs abrogated protection, and the adoptive transfer of Tregs was sufficient to transfer protection against ovalbumin-induced asthma from neonatally infected donors to naive recipients (23).

Here we show that *H. pylori* possesses the ability to profoundly impact the DC maturation process and to convert immature DCs to tolerogenic DCs in vitro and in vivo. *H. pylori*–tolerized DCs are incapable of activating effector functions in naive T cells but become very efficient inducers of FoxP3$^+$ Tregs. DC depletion breaks neonatally induced, *H. pylori*–specific tolerance. We further show that large numbers of semimature DCs are detectable in the chronically infected gastric mucosa of human *H. pylori* carriers and that the tolerogenic properties of *H. pylori*–experienced DCs require the secretion of IL-18 and its direct action on naive T cells. Taken together, our results suggest that *H. pylori* skews the host's immune response toward tolerance over immunity through its direct effects on DCs and that the *H. pylori*/DC interaction forms the mechanistic basis for bacterial persistence and for the protection against asthma that is a hallmark of *H. pylori*–infected individuals.

Results

H. pylori infection prevents DC maturation in a contact-dependent manner. To assess the effects of *H. pylori* infection on DC maturation, we generated immature bone marrow–derived DCs (BM-DCs) and treated the cells with *E. coli* LPS to induce their maturation, either in the absence or presence of *H. pylori*. Flow cytometric analysis of CD80, CD86, and CD40 expression revealed that LPS treatment efficiently induced DC maturation, which was strongly inhibited by the presence of live *H. pylori* (Figure 1, A and B). The infection alone had no effect on the maturation state of DCs (Figure 1, A and B). The expression of DC maturation markers was paralleled by secretion of IL-12 and IL-6 upon LPS treatment, which was also inhibited by *H. pylori* (Figure 1C). Expression of the antiinflammatory cytokine IL-10 was inversely correlated with the other cytokines; its expression was increased by the infection but was not elevated in the LPS-treated samples (Figure 1C). The inhibition of DC maturation did not depend on the presence of a functional cytotoxin-associated gene (*Cag*) pathogenicity island–encoded type IV secretion system, as an *H. pylori* mutant deficient for an essential component of the system, CagE, was as efficient as wild-type bacteria in preventing LPS-induced DC maturation, as assessed by staining for CD80 (Figure 1D) and by ELISA for IL-12p40 (Figure 1E). In contrast, direct contact between wild-type bacteria and the cells was required for this effect, as a transwell filter that prevented attachment of the bacteria also impaired the inhibition of LPS-induced DC maturation (Figure 1, F and G). The observed consequences of *H. pylori* infection were not due to cytotoxic effects of the bacteria on cultured DCs and were at least partially phenocopied by treatment with *H. pylori* extract (Supplemental Figure 1, A–C; supplemental material available online with this article; doi:10.1172/JCI61029DS1). We further alternatively used the TLR2 and TLR9 ligands PAM3Cys and CpG to induce DC maturation; *H. pylori* infection also efficiently prevented CD80 expression and IL-12 secretion induced by these stimuli (Figure 1, H and I, and data not shown). Interestingly, we could not attribute the effects of *H. pylori* on DCs to the C-type lectin receptor DC-specific ICAM3-grabbing nonintegrin (DC-SIGN), which was previously demonstrated to mediate immune escape by *H. pylori* (24). DCs transgenically expressing human DC-SIGN (hDC-SIGN) under the control of the *cd11c* promoter did not differ from wild-type DCs, with respect to the inhibitory effects of *H. pylori* on LPS-induced DC maturation; mice expressing hDC-SIGN (25) further did not differ from wild-type littermates in terms of *H. pylori* colonization levels (Supplemental Figure 1, D–F). Taken together, the results indicate that *H. pylori* has evolved a mechanism of impairing DC function in a manner that depends on direct contact but is independent of the type IV secretion system.

H. pylori exposure generates tolerogenic DCs with the ability to convert naive T cells to FoxP3$^+$ Tregs in a contact- and TGF-β–dependent manner. One of the inherent characteristics of semimature DCs is their ability to convert naive T cells into FoxP3$^+$ Tregs with suppressive activity. To assess whether the exposure to *H. pylori* induces such tolerogenic DCs, we compared the ability of untreated and *H. pylori*–infected BM-DCs to convert naive T cells to FoxP3$^+$ Tregs (Figure 2A). In line with their semimature phenotype (MHCII^{hi}CD-80^{lo}CD86^{lo}CD40^{lo}; Figure 1, A and B; data not shown for MHCII), BM-DCs that had been infected with *H. pylori* induced FoxP3$^+$ Tregs significantly more efficiently than naive BM-DCs, when cultured in the presence of anti-CD3 cross-linking antibody and TGF-β (Figure 2, A and B). Similarly, *H. pylori*–infected BM-DCs loaded with ovalbumin were better able than their naive counterparts to trigger the conversion of ovalbumin-specific (OTII) T cells to FoxP3$^+$ Tregs (Figure 2, C and D). Direct contact between the infected BM-DCs and T cells was required for their efficient conversion to FoxP3$^+$ Tregs, as the separation of both cell populations by a transwell filter abrogated the effect (Figure 2E). Interestingly, DCs isolated from the bone marrow of gene-targeted mice lacking MyD88 or TLR2, an important pattern recognition receptor for *H. pylori* (26, 27), were as capable of Treg conversion upon *H. pylori* exposure as wild-type BM-DCs (Figure 2F). We next immunomagnetically purified CD11c$^+$ DCs from the mesenteric lymph nodes (MLNs) of C57BL/6 mice to more than 80% purity and infected them with live *H. pylori* prior to coculture with naive T cells. Infected MLN-derived DCs (MLN-DCs) were significantly more potent inducers of FoxP3 expression in T cells than naive MLN-DCs in the presence of anti-CD3 cross-linking antibody (Figure 2, G and H) or upon ovalbumin-specific stimulation of OTII T cells (Figure 2, I and J), indicating that the tolerizing effects of *H. pylori* exposure are common to both BM-DC and MLN-DC populations.

H. pylori–experienced DCs are incapable of activating T cell effector responses. To examine whether the exposure of DCs to *H. pylori* not only renders them tolerogenic, but at the same time impairs their ability to activate T cell effector functions and to induce Th1 differentiation, we cocultured *H. pylori*–infected BM-DCs with naive OTII T cells under conditions of anti-CD3 cross-linking or ovalbumin-specific priming. Infected BM-DCs were significantly less able than naive BM-DCs to induce IFN-γ expression and secretion in T cells activated by CD3 cross-linking or ovalbumin priming, as determined by intracellular cytokine staining and ELISA

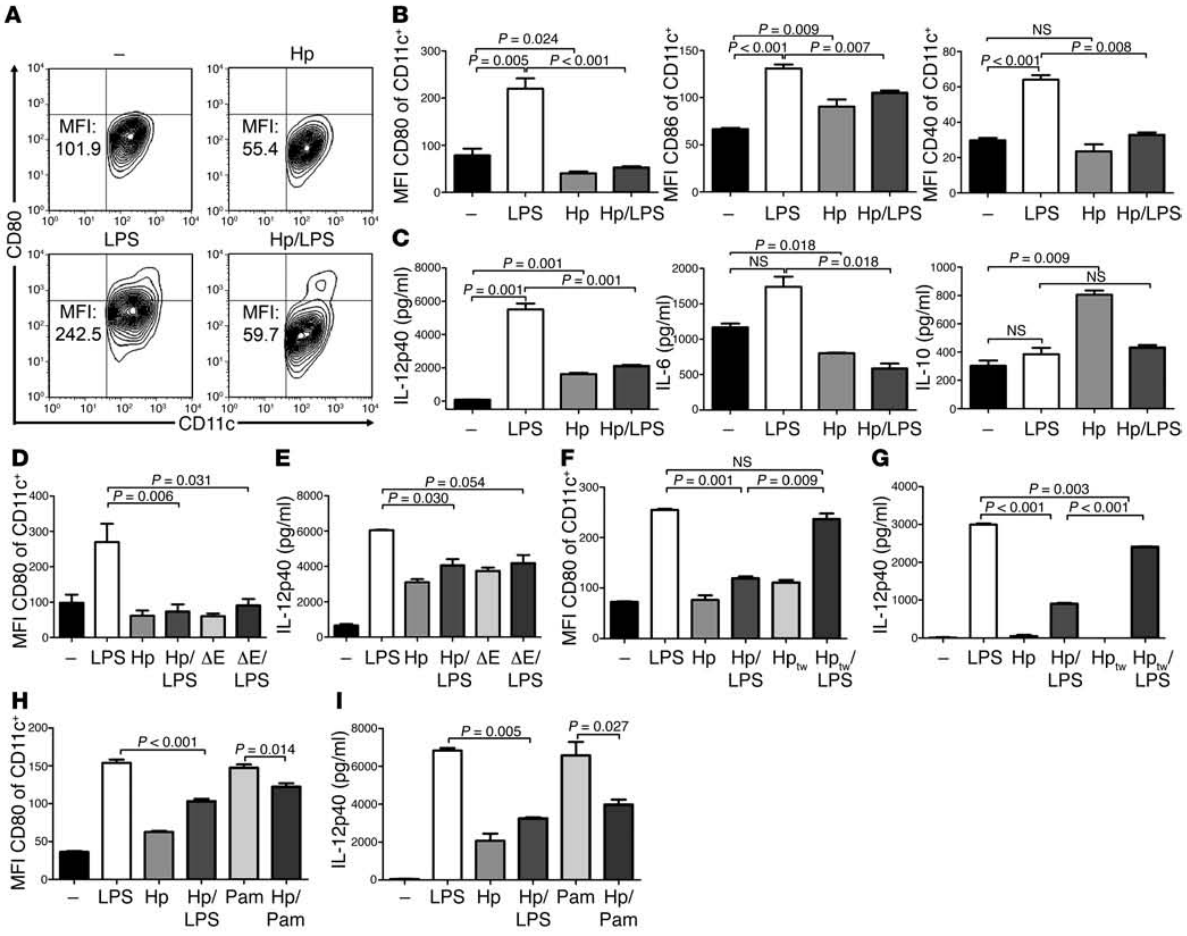


Figure 1

The TLR ligand-induced maturation of DCs is impaired by *H. pylori* infection. (**A**–**C**) BM-DCs were infected with *H. pylori* (Hp) strain PMSS1 at a MOI of 50 and/or treated with 0.5 μg/ml *E. coli* LPS for 16 hours prior to (**A** and **B**) the flow cytometric analysis of CD80, CD86, and CD40 expression and (**C**) the quantification of IL-12p40, IL-6, and IL-10 secretion by ELISA. Representative FACS plots are shown for CD80 in **A**, and the average MFI of CD80, CD86, and CD40 expression of all CD11c+ cells is shown in **B**. (**D** and **E**) BM-DCs were infected with *H. pylori* strain PMSS1 or its isogenic mutant, PMSS1ΔCagE (ΔE), and/or treated with LPS for 16 hours and (**D**) assessed for CD80 expression and (**E**) IL-12p40 secretion. (**F** and **G**) BM-DCs were infected with *H. pylori* strain PMSS1 and/or treated with LPS for 16 hours and (**F**) assessed for CD80 expression and (**G**) IL-12p40 secretion; bacteria were separated from the cells by a transwell (tw) filter where indicated. (**H** and **I**) BM-DCs were infected with *H. pylori* strain PMSS1 and/or treated with 0.5 μg/ml LPS or 5 μg/ml Pam3Cys (Pam) for 16 hours and assessed for (**H**) CD80 expression and (**I**) IL-12p40 secretion. Data are representative of (**D**–**I**) at least 3 and (**A**–**C**) up to 8 independent experiments and are represented as mean ± SEM of triplicate cultures. *P* values were calculated using Student's *t* test.

(Figure 3, A and B). T cell expansion, as assessed by [^{3}H] thymidine incorporation, was impaired as well (Figure 3C). To confirm these results with MLN-DCs, immunomagnetically isolated CD11c+ DCs from the MLNs of naive mice were infected ex vivo and cocultured with OTII-transgenic T cells under conditions of CD3 cross-linking or ovalbumin-specific priming. As observed with the BM-DCs, infected MLN-DCs were significantly less capable of inducing IFN-γ production upon CD3 cross-linking or ovalbumin-specific priming than uninfected MLN-DCs (Figure 3, D–F). The combined results suggest that *H. pylori*–experienced DCs are better inducers of Tregs, but worse inducers of effector T cells, than naive DCs without prior exposure to the bacteria.

H. pylori–experienced DCs fail to induce asthma in a DC-mediated ovalbumin sensitization and challenge model. We and others have shown recently that infection with *H. pylori* protects against allergic asthma in humans (19–22) and in mouse models of the disease (23). A hallmark of infected mice protected against asthma was their pulmonary infiltration with immature, CD11c+ MHCII^{lo} DCs, which were much less abundant in asthmatic mice (23). To determine whether exposure to *H. pylori* indeed generates DCs that are unable to promote asthma, we established a model in which 1 intranasal dose of ovalbumin-loaded DCs was used to sensitize mice to ovalbumin, followed by challenge with aerosolized ovalbumin 2 weeks later. Uninfected, ovalbumin-loaded DCs were compared

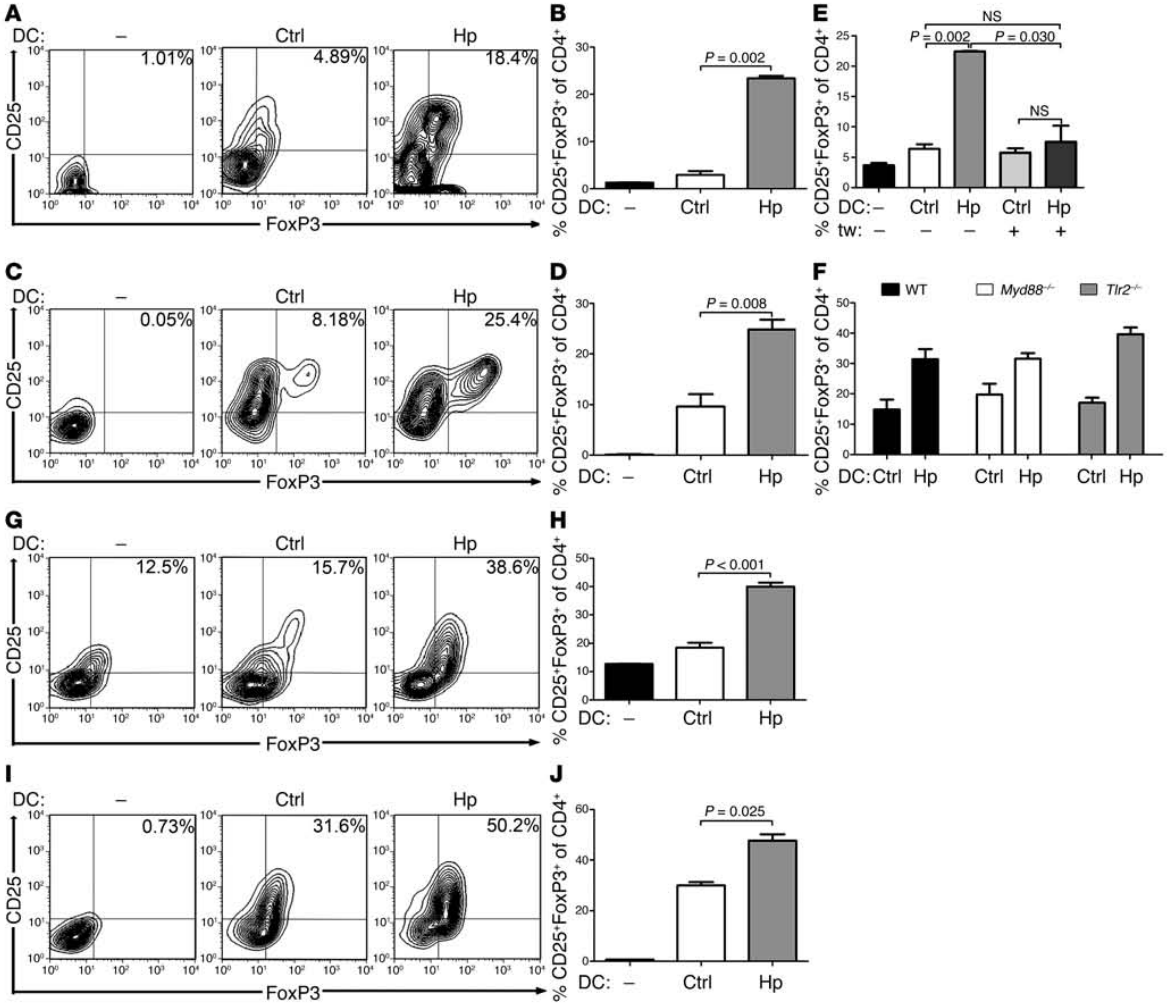


Figure 2

H. pylori–experienced DCs induce FoxP3 expression in naive T cells. (**A** and **B**) BM-DCs were infected with *H. pylori* PMSS1 (MOI 50); after 16 hours, bacteria were killed with antibiotics. DCs were cocultured with immunomagnetically isolated, splenic CD4+CD25– T cells for 3 days in the presence of rTGF-β, rIL-2, and anti-CD3ε mAb prior to flow cytometric analysis of CD4, CD25, and FoxP3 expression. (**A**) Representative plots of the CD4+ gate, (**B**) along with mean ± SEM of triplicate cocultures. T cells cultured in the absence of DCs served as controls (ctrls). (**C** and **D**) BM-DCs were treated as described in **A** and **B** and were additionally loaded with 20 μg/ml ovalbumin prior to coculturing with CD4+CD25– OTII T cells in the presence of rTGF-β and rIL-2. (**C**) Representative CD25 and FoxP3 plots of the CD4+ gate, (**D**) along with mean ± SEM of triplicates. (**E**) BM-DCs and T cells were treated as described in **A**, except that both populations were separated by a transwell filter where indicated. (**F**) Wild-type, *Myd88*$^{-/-}$, and *Tlr2*$^{-/-}$ BM-DCs were treated as described in **A** and **B**. The mean ± SEM of the CD25+FoxP3+ fraction of the CD4+ gate of triplicate cocultures is shown in **E** and **F**. (**G** and **H**) Immunomagnetically isolated, MLN-derived CD11c+ DCs were treated and cocultured with T cells as described in **A** and **B**. (**I** and **J**) MLN-derived CD11c+ DCs were treated and cocultured with OTII T cells as described in **C** and **D**. (**G** and **I**) Representative FACS plots are shown, (**H** and **J**) along with mean ± SEM of triplicate cocultures. Numbers indicate the percentage of FoxP3+CD25+ cells. Data are representative of at least 3 and up to 8 experiments.

with *H. pylori*–infected, ovalbumin-loaded DCs, which had been subjected to antibiotic therapy to kill the bacteria prior to intranasal administration. Additional control groups received DCs that had not been pulsed with antigen. Mice that had received ovalbumin-loaded DCs developed characteristic airway hyperresponsiveness, as assessed upon methacholine inhalation (Figure 4, A and B), and exhibited high levels of peribronchiolar and perivascular inflammation and extensive goblet cell metaplasia (Figure 4, C–E). They further showed high levels of bronchoalveolar infiltration, as assessed by quantification of total cells in the bronchoalveolar lavage fluid (BALF) (Figure 4F). Whereas the BALF from nonasthmatic mice predominantly contained alveolar macrophages, the fraction of macrophages decreased in the BALF of asthmatic recipients of ovalbumin-loaded DCs (Figure 4G). The predomi-

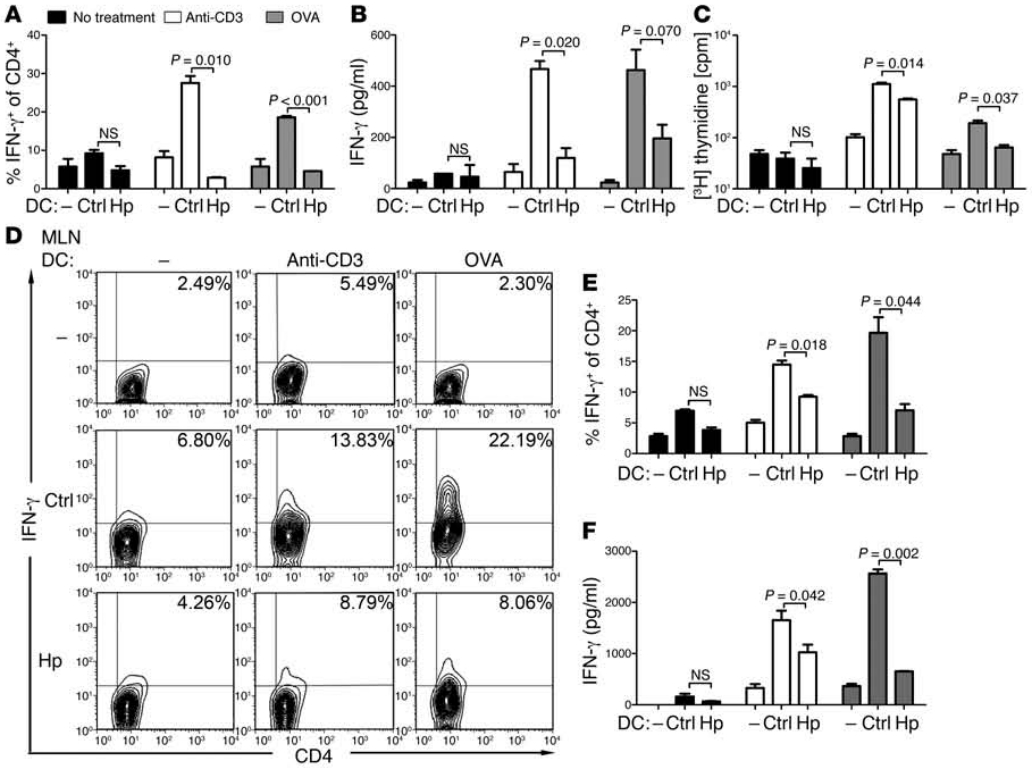


Figure 3

H. pylori infection impairs the ability of DCs to activate T cell effector functions. (**A**–**C**) BM-DCs were infected as described in Figure 2, A and B, and/or loaded with 20 μg/ml ovalbumin prior to coculturing with immunomagnetically isolated, splenic OTII CD4+CD25− T cells for 3 days in the presence of rIL-2. Anti-CD3ε mAb was added where indicated. (**A**) IFN-γ–producing CD4+ T cells were quantified by intracellular cytokine staining, and (**B**) IFN-γ secretion into the supernatant was measured by ELISA. (**C**) Proliferation of parallel cocultures was determined by [3H] thymidine incorporation. T cells cultured without DCs served as controls (–). (**D**–**F**) Immunomagnetically isolated, MLN-derived CD11c+ DCs were infected and/or loaded with 20 μg/ml ovalbumin prior to coculturing with CD4+CD25− T cells in the presence of rIL-2 and anti-CD3ε mAb. (**D**) Representative FACS plots demonstrating intracellular IFN-γ are shown, (**E**) along with mean ± SEM of triplicate cocultures and (**F**) IFN-γ secretion into the supernatant as determined by ELISA. Numbers indicate the percentage of IFN-γ+ cells of the CD4+ gate. All data are representative of at least 3 independent experiments.

nant populations of infiltrating cells in asthmatic mice were lymphocytes, neutrophils, and eosinophils (Figure 4, H–J). None of the described symptoms of asthma were elicited by ovalbumin-loaded DCs that had previously been exposed to *H. pylori* or by DCs not loaded with allergen (Figure 4). In conclusion, the ovalbumin/DC-induced asthma model confirms the inability of infected DCs to prime antigen-specific effector T cell responses.

Neonatally induced H. pylori–specific immune tolerance requires DCs. Hypothesizing that *H. pylori* infection of mice should generate tolerogenic rather than immunogenic DCs in light of our in vitro findings, we infected mice with *H. pylori* PMSS1, a virulent patient isolate that harbors a functional Cag pathogenicity island, establishes persistent infection in mice, and induces the full range of gastric inflammation and preneoplastic pathology that characterizes the *H. pylori*–infected gastric mucosa of susceptible, chronically infected patients (18). In addition to mice infected at 6 weeks of age (i.e., as adults), we included mice that had been infected with *H. pylori* PMSS1 during the neonatal period (i.e., at 7 days of age). Neonatally infected mice develop tolerance rather than immunity to *H. pylori* infection (18); the neonatal infection model is therefore of particular interest in the context of elucidating the tolerogenic properties of *H. pylori*–infected DCs. As the MLNs represent 1 out of 2 documented sites of *H. pylori*–specific T cell priming alongside the Peyer's patches (16, 17, 28, 29), we immunomagnetically isolated MLN-DCs from individual mice from uninfected, neonatally infected, and adult-infected groups and compared them with respect to their ability to induce FoxP3 expression in naive T cells. MLN-DCs from infected mice were significantly more capable of inducing FoxP3 expression in T cells than those isolated from uninfected controls (Figure 5, A and B). The tolerogenic, FoxP3-inducing properties were particularly evident in DCs from neonatally infected, tolerant mice (Figure 5, A and B), which are colonized more densely than their adult-infected counterparts (Figure 5C) due to their lack of CD4+ T cell infiltration into the gastric mucosa (Figure 5D) and their failure to generate Th1 responses to the infection (18). Indeed, DCs from neonatally infected mice were less capable of inducing Th1 effector responses in naive T cells than DCs from mice infected as adults (Figure 5E), underscoring the

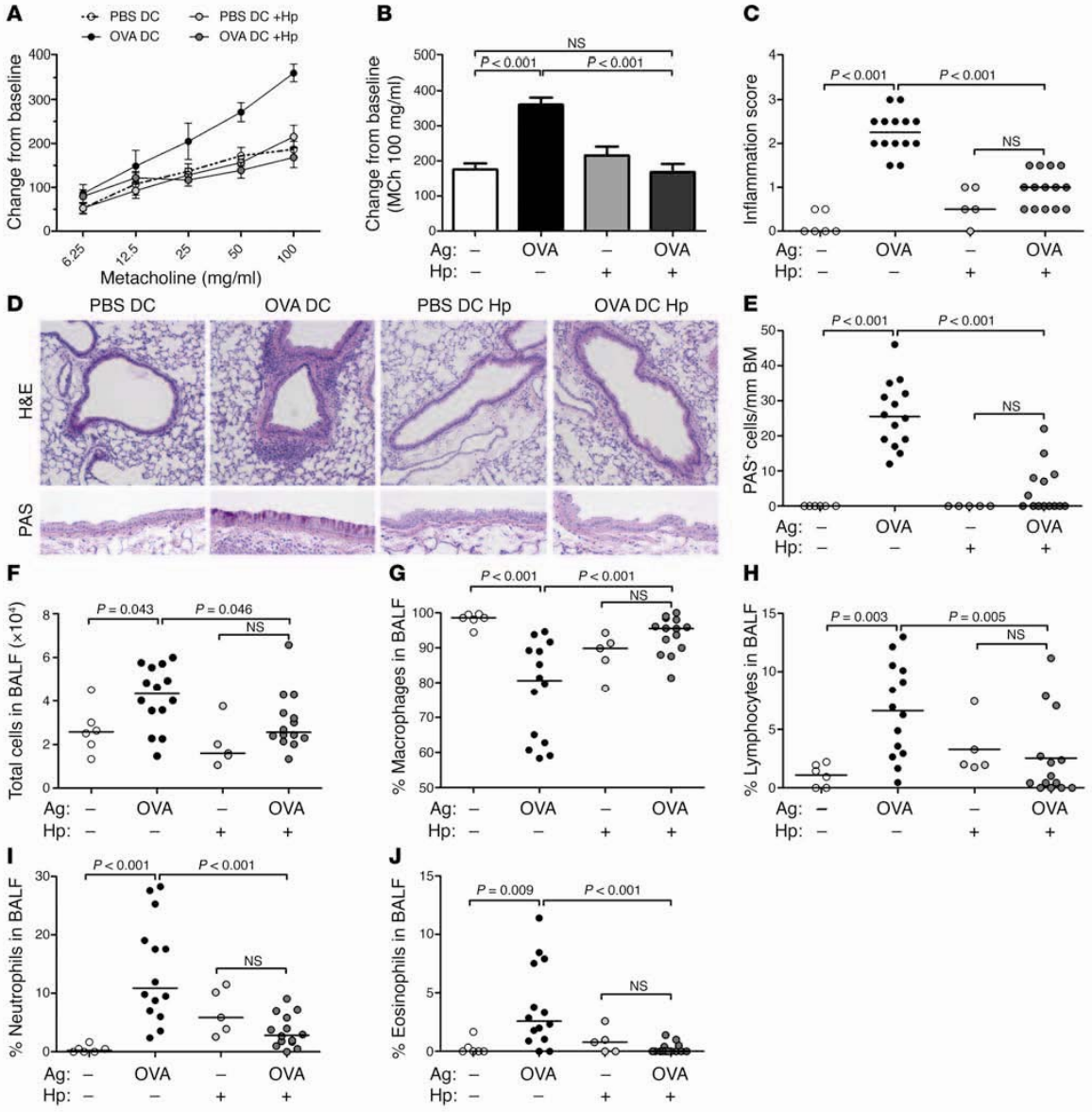


Figure 4

H. pylori–experienced DCs fail to induce asthma-like symptoms in an adoptive transfer model of DC-driven airway hyperresponsiveness. BM-DCs were infected and treated as described in Figure 2, A and B, and/or loaded with either 20 μg/ml ovalbumin (OVA-DC ± Hp [either infected or not infected with *H. pylori*]) or PBS only (PBS-DC ± Hp). 1×10^6 BM-DCs per mouse were administered intranasally for the purpose of ovalbumin-specific sensitization; all mice were challenged 2 weeks later with aerosolized ovalbumin and assessed for the development of airway hyperresponsiveness and tissue inflammation. (**A** and **B**) Airway hyperresponsiveness, (**A**) as assessed by challenge with increasing doses of methacholine and (**B**) the highest dose of 100 mg/ml, respectively. (**C**–**E**) Tissue inflammation and goblet cell metaplasia, as assessed on H&E- and PAS-stained tissue sections. Representative micrographs are shown in **D**. Original magnification, ×100 (H&E); ×400 (PAS). Inflammation and PAS scores are shown in **C** and **E**. (**F**) Total cells contained in 1 ml BALF. Percentages of (**G**) macrophages, (**H**) lymphocytes, (**I**) neutrophils, and (**J**) eosinophils in 1 ml BALF, as determined by differential staining. In the scatter plots shown in **C**, **E**, and **F**–**J**, each data point represents an individual mouse. Data are representative of 3 independent experiments. Horizontal lines represent the median.

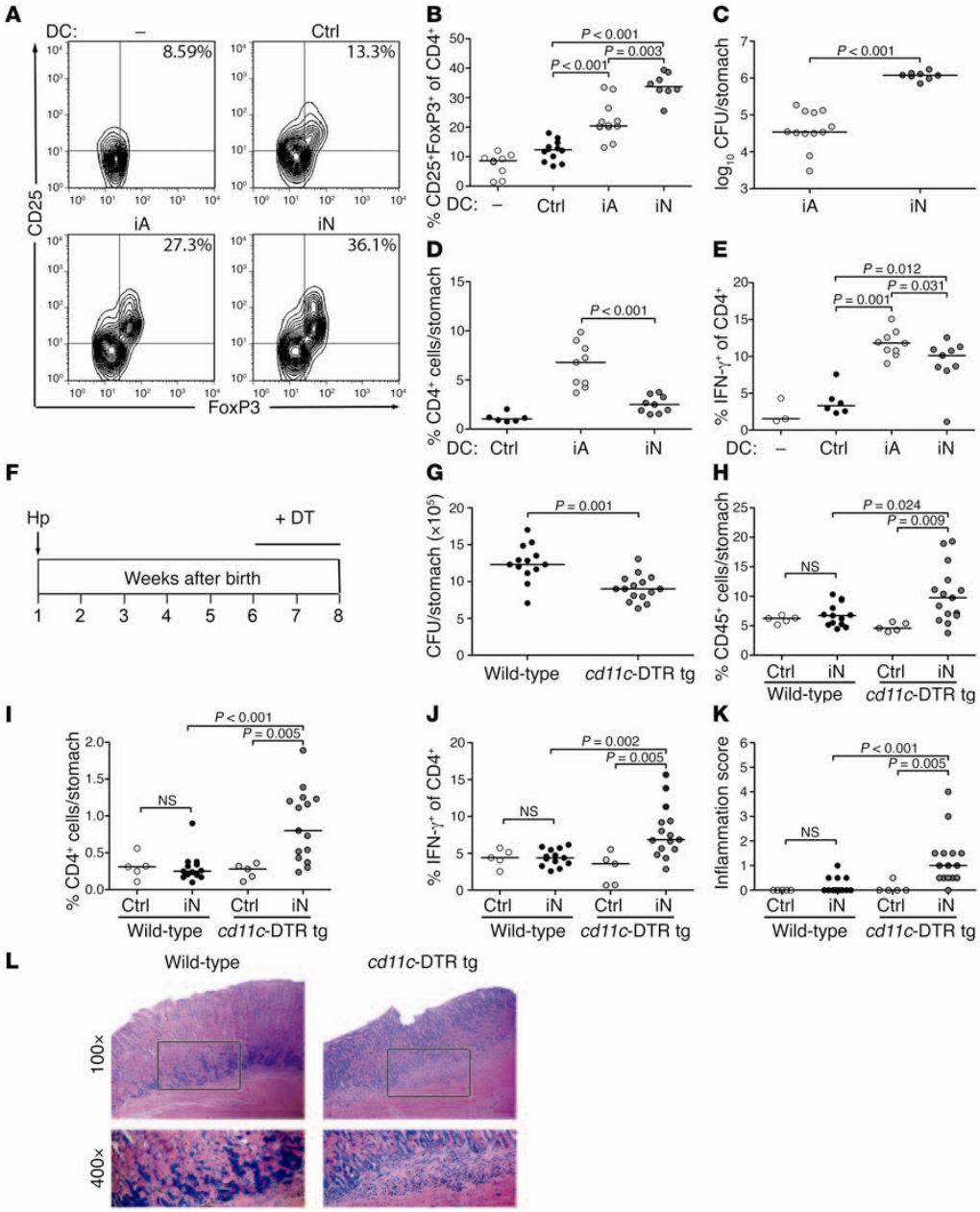


Figure 5

H. pylori–exposed DCs exhibit tolerogenic properties and are required for tolerance in vivo. (**A**–**E**) C57BL/6 mice were infected with *H. pylori* at 7 days (iN) or 6 weeks (iA) of age. Upon sacrifice, CD11c+ MLN-DCs were immunomagnetically isolated; cocultured for 3 days with splenic CD4+CD25− T cells, rTGF-β, rIL-2, and anti-CD3ε mAb; and subjected to flow cytometric analysis of CD4, CD25, and FoxP3 expression. (**A**) CD25 and FoxP3 staining of the CD4+ gate is shown for representative donors and (**B**) quantified for all donors. Numbers indicate the percentage of FoxP3+CD25+ cells. (**C**) *H. pylori* colonization of mice analyzed in **B**. (**D**) CD4+ T cell infiltration into the gastric mucosa of mice shown in **B** and **C**. (**E**) DCs prepared, as described in **A**, were cultured with CD4+CD25− T cells, rIL-2, and anti-CD3ε mAb and subjected to intracellular IFN-γ staining. Each symbol represents an individual donor, and data are pooled from 2 experiments in **B**–**E**. (**F**–**L**) *cd11c*-DTR tg mice and their wild-type littermates were infected at 1 week of age with *H. pylori* strain PMSS1 or remained uninfected. (**F**) All mice received diphtheria toxin during the final 2 weeks of the experiment and were sacrificed 8 weeks after infection. (**G**) Gastric *H. pylori* colonization. (**H** and **I**) Gastric CD45+ leukocyte and CD4+ T cell infiltration. (**J**) Intracellular IFN-γ expression by gastric CD4+ T cells. (**K** and **L**) Gastric histopathology, as assessed on Giemsa-stained sections. Representative micrographs are shown in **L**. Original magnification, ×100 (top); ×200 (bottom). Inflammation scores are shown in **K**. Data in **F**–**L** are pooled from 3 experiments. Horizontal lines indicate the medians.

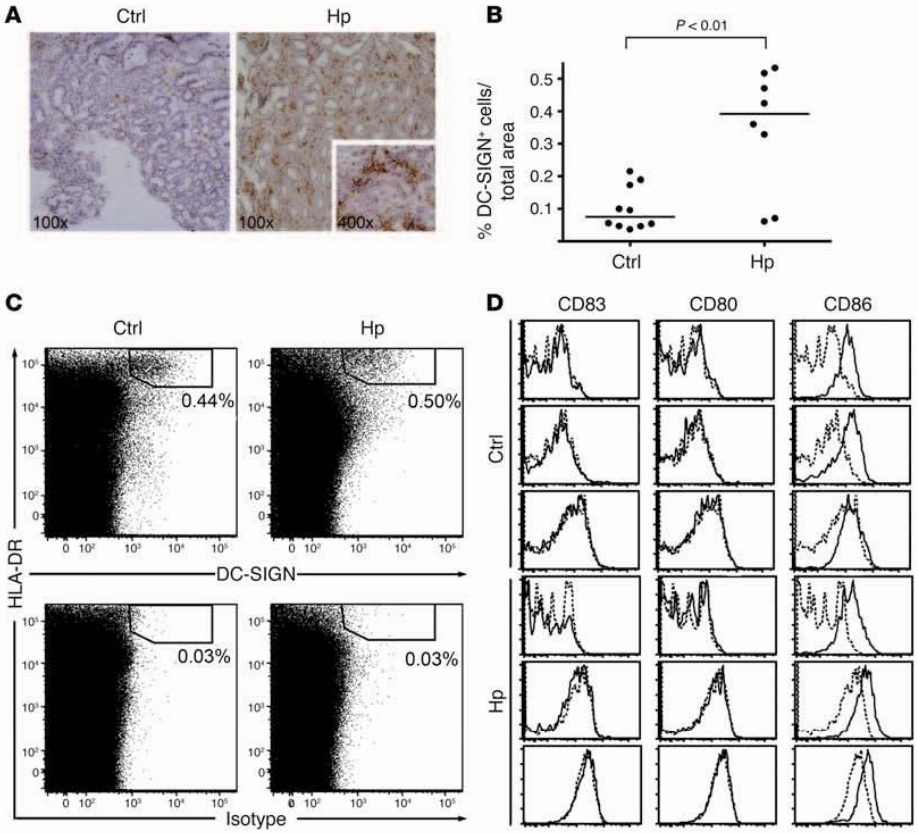


Figure 6

Human gastric DCs exhibit a semimature phenotype in infected patients. (**A** and **B**) Antral biopsies were collected from healthy uninfected and *H. pylori*–infected individuals, and expression of DC-SIGN was determined by immunohistochemical staining. (**A**) Representative stainings are shown (original magnification, ×100; ×400 [inset]), (**B**) along with frequencies of DC-SIGN$^+$ DCs, expressed as percentage of stained area in the gastric mucosa. Each symbol indicates an individual donor; horizontal lines represent medians. (**C**) Gastric DCs in lamina propria single cell preparations from uninfected and *H. pylori*–infected patients undergoing gastrectomy were identified by flow cytometry as HLA-DR^{hi}DC-SIGN$^+$ cells. Lower dot plots show staining with an isotype control for the DC-SIGN antibody. The percentages indicate cell frequencies among total live (7AAD$^-$) cells. (**D**) Flow cytometric analysis of the immunophenotype of gastric HLA-DR^{hi}DC-SIGN$^+$ DCs. The solid line in histograms shows staining with the indicated antibody, and the dotted line shows staining with isotype controls. Data are shown for 3 uninfected and 3 *H. pylori*–infected individuals.

mutual exclusion of tolerogenic and immunogenic properties in a given DC population. To obtain functional evidence that DCs are required for the development and maintenance of neonatally acquired immunological tolerance to *H. pylori* infection, we infected mice that transgenically express the diphtheria toxin receptor under the control of the CD11c promoter (*cd11c*-DTR tg mice) at 1 week of age. At 6 weeks after infection, the *cd11c*-DTR tg mice as well as their nontransgenic, infected littermates and respective uninfected controls of both genotypes received 3 weekly doses of diphtheria toxin for 2 weeks (Figure 5F), resulting in the depletion of approximately 40%–50% of DCs in the gastric mucosa, MLNs, and spleen (Supplemental Figure 2 and data not shown). DC-depleted mice were colonized at significantly lower levels (Figure 5G) and exhibited higher levels of leukocyte and CD4$^+$ T cell infiltration into the gastric mucosa than their DC-replete, infected littermates and uninfected controls (Figure 5, H and I). A higher proportion of CD4$^+$ gastric mucosal T cells expressed IFN-γ in the DC-depleted, infected mice than in the other 3 groups (Figure 5J). The infiltration of leukocytes into the gastric mucosa of DC-depleted, infected mice was histologically evident as mild to moderate gastritis, a feature that is never observed in neonatally infected, fully tolerant mice at such early time points after infection (Figure 5, K and L). In summary, the depletion of CD11c$^+$ DCs in the *cd11c*-DTR tg model reveals that the maintenance of *H. pylori*–specific immunological tolerance requires DCs, a finding that may be explained by the predominantly tolerogenic properties of *H. pylori*–experienced DCs in vitro and in vivo.

DCs infiltrating the gastric mucosa of H. pylori–infected patients express HLA-DR but lack costimulatory molecules. To examine the recruitment and localization of DCs to the gastric mucosa of *H. pylori*–infected individuals, we examined antral biopsies collected at endoscopy from *H. pylori*–infected and uninfected volunteers. Cryosections from antral biopsies were immunostained for DC-SIGN, a marker for human DCs (30). The large majority of DC-SIGN$^+$ DCs were

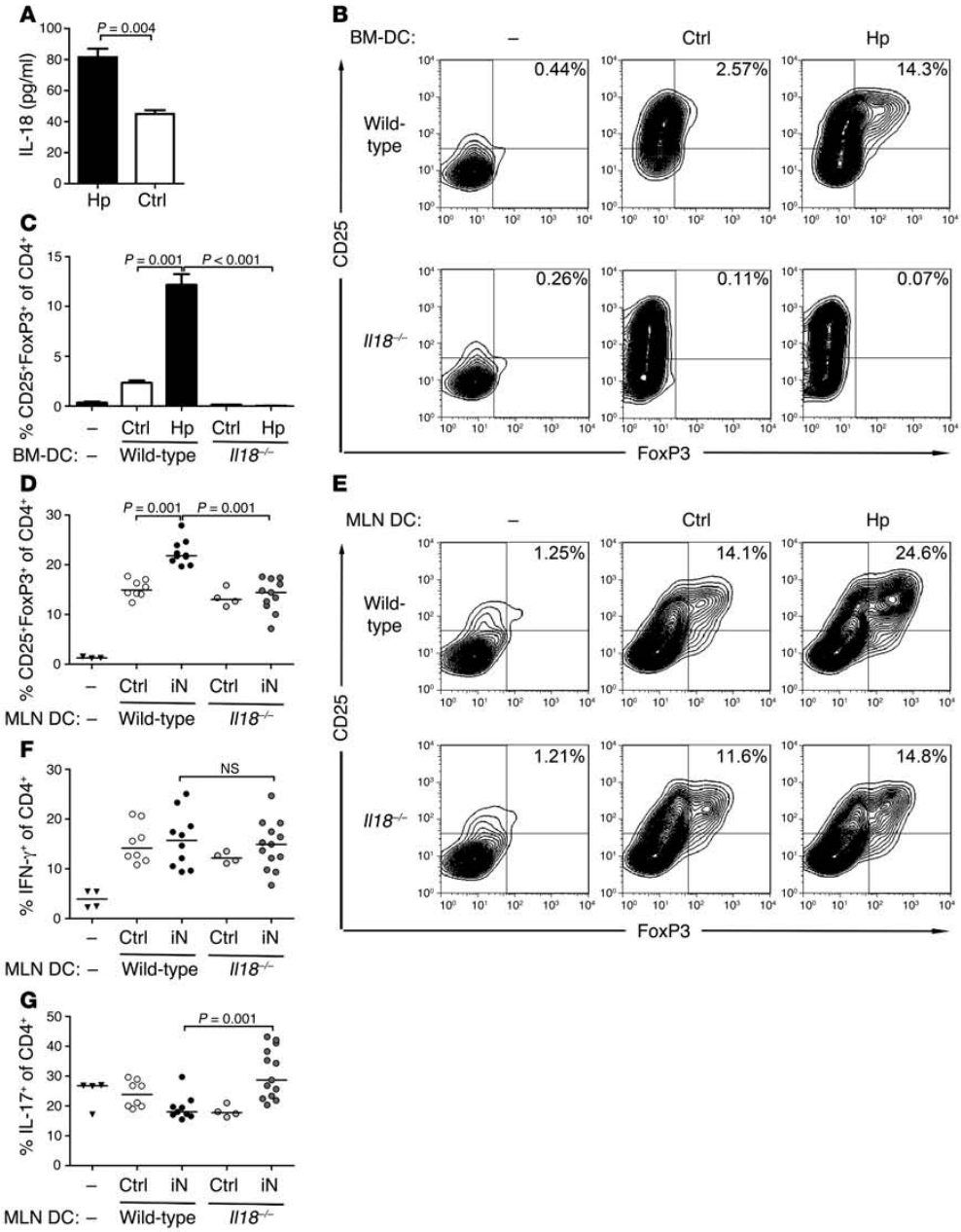


Figure 7

Treg induction by tolerogenic DCs requires IL-18. (**A**) Wild-type BM-DCs were infected with *H. pylori* PMSS1 (MOI 50), and IL-18 secretion was assessed by ELISA. (**B** and **C**) Wild-type and *Il18*$^{-/-}$ BM-DCs were infected as described in **A**, and cocultured at a 1:2 ratio with immunomagnetically isolated, splenic OTII CD4+CD25− T cells for 3 days in the presence of rTGF-β, rIL-2, and anti-CD3ε mAb prior to the flow cytometric analysis of CD4, CD25, and FoxP3 expression. (**B**) Representative FACS plots of the CD4+ gate are shown, (**C**) along with mean ± SEM of triplicate cocultures. (**D**–**G**) C57BL/6 and BL/6.*Il18*$^{-/-}$ mice were infected at 7 days (iN) of age with 1 orogastric dose of *H. pylori* or remained uninfected. Upon sacrifice after 4 weeks after infection, CD11c+ DCs were immunomagnetically isolated from single cell MLN suspensions of individual mice and cocultured for 3 days with splenic CD4+CD25− T cells at a 1:2 ratio in the presence of rIL-2 and anti-CD3ε mAb and (**D** and **E**) with or (**F** and **G**) without rTGF-β. (**D** and **E**) Cocultures were subjected to flow cytometric analysis of CD4, CD25, and FoxP3 expression (representative mice are shown in **E**, and the quantification of all mice is shown in **D**) or (**F** and **G**) CD4 and IFN-γ or IL-17 expression. Data shown are representative of at least 3 independent (**A**–**C**) in vitro and (**D**–**G**) in vivo experiments. Horizontal lines represent the median. (**B** and **E**) Numbers indicate the percentage of FoxP3+CD25+ cells.

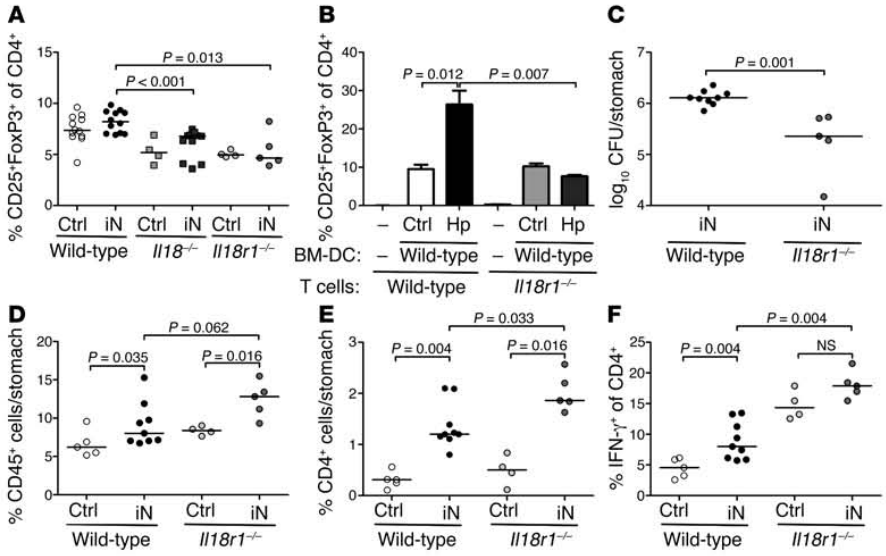


Figure 8

Treg differentiation and the development of *H. pylori*–specific tolerance requires IL-18 signaling in vivo. (**A**) Single cell MLN preparations from individual C57BL/6, BL/6.*Il18*$^{-/-}$, and BL/6.*Il18r1*$^{-/-}$ mice neonatally infected with *H. pylori* were subjected to flow cytometric analysis of CD4, CD25, and FoxP3 expression and compared with respective uninfected controls. Each data point represents an individual mouse; horizontal lines indicate medians. (**B**) Wild-type BM-DCs were infected with *H. pylori* PMSS1 (MOI 50) and cocultured at a 1:2 ratio with wild-type or *Il18r1*$^{-/-}$ CD4$^+$CD25$^-$ T cells for 3 days in the presence of rTGF-β, rIL-2, and anti-CD3ε mAb prior to analysis of CD4, CD25 and FoxP3 expression. Mean ± SEM of triplicate cocultures are shown. (**C**–**F**) Wild-type C57BL/6 and BL/6.*Il18r1*$^{-/-}$ mice were neonatally infected with *H. pylori* and sacrificed 4 weeks after infection. (**C**) Gastric *H. pylori* colonization. (**D** and **E**) Gastric mucosal CD45$^+$ leukocyte and CD4$^+$ T cell infiltration. (**F**) IFN-γ expression by stomach-infiltrating CD4$^+$ T cells, as determined by intracellular cytokine staining. Horizontal lines represent the median.

found scattered in the lamina propria of both uninfected and *H. pylori*–infected individuals (Figure 6A), and some DCs were positioned just underneath the epithelium. The frequency of DC-SIGN$^+$ DCs was significantly higher in *H. pylori*–infected individuals compared with that in their uninfected counterparts (Figure 6B). To immunophenotypically characterize the DC-SIGN$^+$ DCs in the gastric mucosa of *H. pylori*–infected patients, we obtained gastric tissue from a set of 8 patients undergoing gastrectomy at Sahlgrenska University Hospital (of which 4 were *H. pylori* infected and 4 were uninfected). Lamina propria single cell suspensions were prepared, and DCs were identified by flow cytometry among live cells (7AAD$^-$) based on their expression of high levels of HLA-DR together with DC-SIGN (Figure 6C). The HLA-DR^{hi}DC-SIGN$^+$ DCs expressed the DC marker CD11c (data not shown) but no or only little CD80, CD83, or CD86 (Figure 6D). Furthermore, there were no consistent differences in DC phenotype when comparing *H. pylori*–infected and uninfected patients (Figure 6D). In summary, the results suggest that human gastric DCs, despite being actively recruited in large numbers to the *H. pylori*–infected mucosa, fail to upregulate activation markers and thus appear to retain a semimature phenotype. This observation may explain the ability of *H. pylori* to persist in the human gastric mucosa.

DC-intrinsic IL-18 expression is required for the conversion of naive T cells to FoxP3$^+$ Tregs in vitro. As part of our ongoing efforts to characterize the molecular players contributing to *H. pylori*–associated gastric immunopathology, manifesting as gastric inflammation, atrophy, epithelial hyperplasia, and intestinal metaplasia, we discovered that *Il18*$^{-/-}$ mice were better able to control experimental *Helicobacter* infections than wild-type controls and, as a consequence, developed strongly accelerated and aggravated gastric immunopathology (Supplemental Figure 3, A and B). Based on these findings, we speculated that IL-18 may be required for the efficient conversion of naive T cells to FoxP3$^+$ Tregs by *H. pylori*–experienced DCs in vitro. To test this notion, we first examined whether IL-18 secretion is induced upon *H. pylori* infection of BM-DCs, which was indeed the case (Figure 7A). We then compared the efficiency of FoxP3 induction by *H. pylori*–infected wild-type and *Il18*$^{-/-}$ BM-DCs under conditions of anti-CD3 cross-linking and ovalbumin-specific priming. As demonstrated above (Figure 2), wild-type BM-DCs that had been infected with *H. pylori* induced FoxP3 expression in cocultured naive OTII T cells more efficiently than control BM-DCs, independent of whether the T cells were activated by anti-CD3 cross-linking (Figure 7, B and C) or ovalbumin (Supplemental Figure 3, C and D). Interestingly, infected *Il18*$^{-/-}$ BM-DCs exhibited a clear defect in FoxP3 induction under both coculture conditions (Figure 7, B and C, and Supplemental Figure 3, C and D). In line with a reported role for IL-18 in Th1 differentiation in vitro, both uninfected and infected *Il18*$^{-/-}$ DCs failed to induce IFN-γ expression in cocultured naive T cells (Supplemental Figure 4, A–D). We next sought to assess a possible role for mature IL-18 in generating tolerogenic DCs in the course of neonatal infection with *H. pylori* in vivo. As demonstrated above (Figure 5), immunomagnetically isolated MLN-DCs from neonatally infected wild-type mice were significantly better inducers of FoxP3 expression than MLN-DCs from naive mice (Figure 7, D and E). In contrast, MLN-DCs from infected *Il18*$^{-/-}$ mice did not induce FoxP3 expression in T cells any more efficiently than MLN-DCs from naive *Il18*$^{-/-}$ mice (Figure 7, D and E). Interestingly, whereas Treg differentiation induced by MLN-DCs was thus severely impaired due to the lack of DC-intrinsic IL-18 production (Figure 7, D and E), DCs from infected *Il18*$^{-/-}$ mice were equally good as wild-type DCs at inducing Th1 differentiation in this system (Figure 7F) and significantly better at inducing Th17 differentiation than wild-type DCs (Figure 7G). The combined results show that IL-18 production is induced in DCs upon infection with *H. pylori* and suggest a key role for DC-derived IL-18 in skewing T cell differentiation away from Th17 and toward Treg responses.

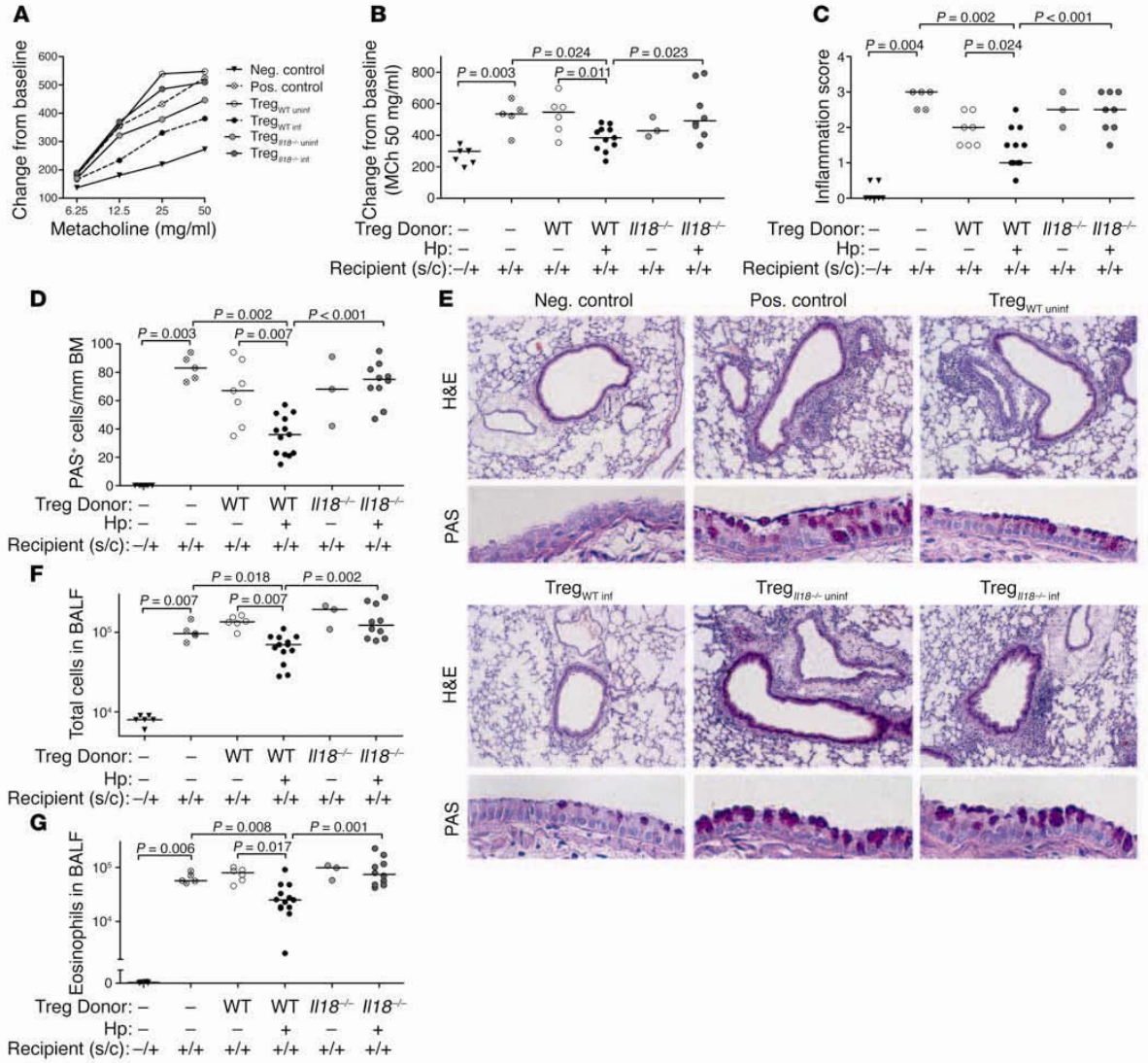


Figure 9

IL-18 signaling is required for the differentiation of functional Tregs in vivo. Wild-type C57BL/6 mice were sensitized with 2 i.p. doses of alum-adjuvanted ovalbumin prior to challenge with aerosolized ovalbumin 2 weeks after the last sensitization. Four groups of sensitized recipients received 250,000 immunomagnetically isolated CD4+CD25+ T cells isolated from the pooled MLNs of uninfected or neonatally infected wild-type C57BL/6 or BL/6.*Il18*$^{-/-}$ donors (4–6 per group) 1 day before the first challenge. Negative controls were challenged without prior sensitization. (**A** and **B**) Airway hyperresponsiveness, (**A**) as assessed by challenge with increasing doses of methacholine and (**B**) the highest dose of 50 mg/ml, respectively. (**C**–**E**) Tissue inflammation and goblet cell metaplasia, as assessed on H&E- and PAS-stained tissue sections. Representative micrographs are shown in **E**. Original magnification, ×100 (H&E); ×400 (PAS). Inflammation and PAS scores are shown in **C** and **D**, respectively. (**F**) Total cells contained in 1 ml BALF. (**G**) Eosinophils in 1 ml BALF. Horizontal lines indicate medians. s/c, sensitized/challenged.

IL-18 signaling is required for Treg differentiation and H. pylori–specific tolerance in vivo. Hypothesizing based on the above results that Treg numbers should consequently differ between the MLNs of wild-type and *Il18*$^{-/-}$ mice, we quantified the proportion of FoxP3+CD25+ cells in the MLN CD4+ T cell population of neonatally infected wild-type and *Il18*$^{-/-}$ mice in comparison with that of uninfected controls of both genetic backgrounds. Whereas the infection induced an increase in MLN Tregs in wild-type mice, this was not observed in *Il18*$^{-/-}$ animals, resulting in significantly lower Treg numbers in infected *Il18*$^{-/-}$ animals compared with infected wild-type animals (Figure 8A). Interestingly, the same clear trend was observed in mice lacking the IL-18 receptor (IL-18R; Figure 8A), which also exhibited significantly lower Treg counts upon neonatal infection than wild-type mice. Hypothesizing that DC-derived

IL-18 acts directly on T cells to facilitate their conversion to FoxP3$^+$CD25$^+$ Tregs, we compared naive wild-type and *Il18r1*$^{-/-}$ T cells, with respect to their ability to upregulate FoxP3 upon coculture with *H. pylori*–experienced BM-DCs. Indeed, *Il18r1*$^{-/-}$ T cells showed a strongly reduced ability to upregulate FoxP3, indicating that IL-18 signaling in T cells is required for efficient Treg conversion in vitro (Figure 8B). We next examined the ability of *Il18r1*$^{-/-}$ mice to develop tolerance to *H. pylori* as a consequence of experimental infection during the neonatal period. Neonatally infected *Il18r1*$^{-/-}$ mice exhibited significantly lower colonization levels than wild-type mice infected at the same age (Figure 8C), which was accompanied by higher gastric leukocyte and T cell infiltration (Figure 8, D and E) and stronger gastric production of IFN-γ and IL-17, as determined by intracellular cytokine staining and/or qPCR (Figure 8F and Supplemental Figure 5). We conclude from these results that T cell–intrinsic IL-18 signaling is essential for Treg differentiation in vitro and in vivo and is also required for the generation of Treg-mediated, neonatally acquired tolerance to *H. pylori* infection.

CD4$^+$CD25$^+$ T cells isolated from neonatally infected wild-type mice but not Il18$^{-/-}$ *or Il18r1*$^{-/-}$ *mice suppress allergen-induced asthma.* Given the requirement for DC-derived IL-18 in Treg differentiation in vitro and in vivo, we hypothesized that CD4$^+$CD25$^+$ T cells immunomagnetically isolated from the MLNs of neonatally infected *Il18*$^{-/-}$ or *Il18r1*$^{-/-}$ mice should lack suppressive activity in a murine model of experimentally induced allergic airway disease. Having shown earlier that neonatal infection of wild-type mice with *H. pylori* generates Tregs with highly suppressive activity in the asthma model (23), we compared immunomagnetically isolated CD4$^+$CD25$^+$ T cells from neonatally infected wild-type, *Il18*$^{-/-}$, or *Il18r1*$^{-/-}$ animals, with respect to their ability to inhibit experimentally induced asthma (Figure 9 and Supplemental Figure 6). Mice were sensitized i.p. with alum-adjuvanted ovalbumin and subsequently challenged by 3 consecutive daily exposures to aerosolized ovalbumin. Groups of mice were intravenously administered CD4$^+$CD25$^+$ T cells isolated from either neonatally infected wild-type, *Il18*$^{-/-}$, or *Il18r1*$^{-/-}$ animals or from age-matched uninfected controls of each genotype 1 day prior to the first challenge. All mice were examined with respect to clinical and histopathological parameters of asthma development 2 days after the last challenge. Sensitized, challenged mice exhibited increased airway resistance compared with nonsensitized controls, as assessed by methacholine challenge, which could be prevented by CD4$^+$CD25$^+$ T cells from infected wild-type animals but not infected *Il18*$^{-/-}$ animals (Figure 9, A and B) or *Il18r1*$^{-/-}$ animals (Supplemental Figure 6, A and B). CD4$^+$CD25$^+$ T cells from uninfected control groups did not prevent allergen-induced airway resistance (Figure 9, A and B). Similarly, the lung inflammation and PAS$^+$ goblet cell metaplasia that are hallmarks of asthma in this model were prevented by CD4$^+$CD25$^+$ T cells from infected wild-type mice but not infected *Il18*$^{-/-}$, *Il18r1*$^{-/-}$, or uninfected mice (Figure 9, C–E, and Supplemental Figure 6, C and D). A quantitative analysis of allergen-induced bronchoalveolar inflammation and eosinophilia confirmed these findings (Figure 9, F and G, and Supplemental Figure 6, E and F). Interestingly, quantitative RT-PCR analysis of IL-10 expression performed on freshly purified CD4$^+$CD25$^+$ T cells revealed that T cells isolated from infected wild-type mice, but not infected *Il18*$^{-/-}$ mice, expressed copious amounts of IL-10 compared with CD4$^+$CD25$^+$ T cells from the corresponding uninfected donors (Supplemental Figure 6, G and H), implying that this cytokine may be key in asthma protection by *H. pylori*–induced Tregs. Overall, the results suggest that Treg differentiation and function is severely impaired under conditions of IL-18 or IL-18R deficiency, underscoring the importance of this cytokine in Treg biology.

Discussion

H. pylori infection is acquired during early childhood and typically persists for life (31). Chronic infection with *H. pylori* causes gastritis and predisposes to gastric cancer but has also been associated epidemiologically with protection against asthma (19, 20, 22), multiple sclerosis (32), and inflammatory bowel disease (33). The beneficial effects of the infection are particularly evident in the pediatric population, with respect to both asthma and colitis incidence (21, 34). Experimental infection with *H. pylori* during the neonatal period recapitulates the protective effects against asthma in mouse models of the disease (23). We show here that the exposure of DCs to *H. pylori* had profound effects on the properties of these cells in vitro and in vivo. *H. pylori*–experienced BM-DCs or MLN-DCs lose their ability to mature into fully immunogenic, stimulatory antigen-presenting cells and to activate effector functions in naive T cells. Instead, *H. pylori* exposure triggers tolerogenic properties in DCs, which become efficient inducers of Tregs in vitro and suppress *H. pylori* clearance and the associated immunopathology in vivo. Our results explain why the systemic depletion of DCs improves the immunological control of *H. pylori* in naive mice and enhances vaccine-induced protective immunity in immunized mice (12). Our BM-DC results suggest that direct contact between the bacteria and DCs is required for the observed effects, whereas a functional Cag pathogenicity island–encoded type IV secretion system is dispensable. Semimature DCs with high expression of HLA-DR but low expression of CD80, CD83, and CD86 constitute the predominant DC population in the stomachs of chronically infected humans; this finding is particularly intriguing, as DCs are actively recruited to the infected gastric mucosa but obviously fail to mature and, presumably, to acquire immunogenic/stimulatory properties. Our findings are reminiscent of a recent study by Robinson et al., who found that patients with peptic ulcer disease exhibited stronger Th1 and Th2 responses to *H. pylori* than asymptomatic carriers, whereas the latter group predominantly mounted Treg responses to the infection (35). IL-10–expressing Tregs were particularly abundant in the gastric mucosa of the asymptomatic carriers compared with patients with peptic ulcer disease; interestingly, mucosal IL-10 levels were directly correlated with bacterial densities, with asymptomatic carriers showing high IL-10 expression and receiving the highest colonization scores and patients with peptic ulcer disease showing low IL-10 expression and receiving comparatively low colonization scores (35). This interesting association argues that strong gastric Treg responses (perhaps induced by tolerogenic DCs) ensure persistent infection on the one hand and prevent severe immunopathology on the other.

Several previous reports have highlighted the fact that *H. pylori* possesses few, if any, PAMPs that might activate innate immune responses by binding to surface exposed, endosomal, or cytoplasmic pattern recognition receptors (27, 36, 37). *H. pylori* flagellin is a poor ligand of TLR5 (36), and the bacterium's LPS consists predominantly of the biologically inactive tetra-acylated lipid A variety (38). Whereas TLR2 ligands clearly exist in *H. pylori* and related *Helicobacter* species (26, 27), the net effects of TLR2 ligation are antiinflammatory rather than proinflammatory (27). We show here that the DC-derived cytokine IL-18 plays a crucial role in the

tolerogenic activity of *H. pylori*–experienced DCs in vitro and in vivo. In contrast to wild-type cells, BM-DCs derived from *Il18*$^{-/-}$ mice failed to induce FoxP3 expression in naive T cells upon exposure to *H. pylori*; more importantly, *Il18*$^{-/-}$ MLN-DCs from neonatally infected mice exhibited the same defect in Treg induction relative to their wild-type counterparts. DC-derived IL-18 appears to act directly on T cells and to promote their conversion to Tregs: IL-18R–deficient T cells failed to upregulate FoxP3 upon coculture with tolerogenic DCs, and both IL-18– and IL-18R–deficient mice exhibited strongly decreased overall Treg numbers in the draining MLNs. Furthermore, CD4$^+$CD25$^+$ MLN T cells isolated from both gene-targeted strains are less capable of suppressing allergic airway disease in an allergen-induced sensitization and challenge model. All available data thus suggest that IL-18 contributes to *H. pylori* persistence and fine-tunes the balance between infection control on the one hand and excessive immunopathological T cell responses on the other. The results are confirmed in vivo by our observation that *Il18r1*$^{-/-}$ mice failed to develop immune tolerance to neonatal infection with *H. pylori*. Our data are in line with 2 recent studies demonstrating a protective role for IL-18, produced upon activation of the NLRP3 inflammasome, in DSS-induced colitis (39, 40). In this model, lack of expression of NLRP3, caspase-1, and ASC in intestinal epithelial cells rendered mice hypersusceptible to DSS-induced colitis (39, 40).

As mentioned earlier, the protection against asthma is mediated by *H. pylori*–induced Tregs in experimentally infected mice: asthma protection is abrogated by the systemic depletion of Tregs and can be adoptively transferred from infected donors to uninfected recipients via Tregs (23). An interesting characteristic of neonatally infected mice that have been subjected to an asthma-inducing protocol is their pulmonary infiltration by highly suppressive Tregs and by semimature DCs (23). Our finding that *H. pylori*–exposed DCs are incapable of sensitizing mice to ovalbumin when administered intranasally makes it seem likely that *H. pylori*–mediated asthma protection is a direct consequence of the reprogramming of DCs in vivo. However, as DCs are not known to migrate between the mucosal surfaces of the lung and gastrointestinal tract, it appears likely that highly mobile Tregs are induced in the MLNs and then migrate to the airways, in which they exert their suppressive activity and protect against allergen-induced asthma. DCs and Tregs efficiently regulate one another in a negative feedback loop, promoting a tolerogenic, immunosuppressive environment. For instance, Onishi et al. reported that FoxP3$^+$ Tregs form aggregates on DCs, thereby actively downregulating their costimulatory molecules and maturation markers and impairing the ability of DCs to activate antigen-specific, naive T cells (41). In summary, we show here that *H. pylori* possesses the distinct ability to reprogram DCs toward a tolerogenic phenotype in vitro and in vivo, a process that ensures persistence of the bacteria in the host and may cross-protect against chronic inflammatory and autoimmune diseases. The semimature phenotype of lamina propria DCs that is a hallmark of the gastric mucosa of asymptomatic human *H. pylori* carriers likely represents the human cellular counterpart of the murine tolerogenic DC population.

Methods

Animal experimentation; bronchoalveolar lavage, lung, and gastric histopathology; and assessment of H. pylori colonization. C57BL/6 wild-type, BL/6.*Tlr2*$^{-/-}$, BL/6.*Myd88*$^{-/-}$, OT II TCR transgenic, and *cd11c*-DTR tg mice were originally purchased from Charles River Laboratories; BL/6.*Il18*$^{-/-}$, BL/6.*Il18r1*$^{-/-}$, and *cd11c*-hDC-SIGN transgenic mice were obtained from Wolf-Dietrich Hardt (Federal Institute of Technology, Zürich, Switzerland), Burkhard Becher (University of Zürich), and Tim Sparwasser (Medical School, University of Hannover, Hannover, Germany). All mice were bred at a University of Zurich specific pathogen–free facility. Mice were housed in individually ventilated cages. Mixed gender groups were infected at either 7 days or 6 weeks of age with 1 orogastric dose of approximately 2×10^7 CFUs *H. pylori* PMSS1 (18). In vivo depletion of DCs was achieved by i.p. injections of 4 ng diphtheria toxin per g of body weight at 2-day intervals for 2 weeks. For asthma induction, mice were sensitized either by 2 i.p. injections of 20 μg ovalbumin (Sigma-Aldrich) emulsified in 2.25 mg aluminum hydroxide (Alum Imject; Pierce) with a 2-week interval or by intranasal delivery of 1×10^6 BM-DCs loaded overnight (o/n) with 20 μg/ml ovalbumin (Sigma-Aldrich). Irrespective of the mode of sensitization, mice were challenged with 1% aerosolized ovalbumin using an ultrasonic nebulizer (NE-U17; Omron) for 20 minutes daily on days 14, 15, and 16 after (second) sensitization. Airway resistance measurements were performed on anesthetized, intubated, and mechanically ventilated mice (FlexiVent, Scireq) in response to increasing doses of inhaled methacholine, as described previously (42). Lungs were lavaged via the trachea with 1 ml PBS. BALF cells were counted using trypan blue dye exclusion. Differential cell counts of lymphocytes, neutrophils, and eosinophils were performed on cytocentrifuged preparations stained with the Microscopy Hemacolor Set (Merck). Lungs were fixed by inflation and immersion in 10% formalin and embedded in paraffin. Lung tissue sections were stained with H&E and PAS and were examined on a BX40 Olympus microscope in blinded fashion by 2 independent experimenters. Peribronchial inflammation was scored on a scale from 0 to 4 on 5 randomly chosen areas per slide, as described previously (43). PAS-positive goblet cells were quantified per 1 mm of basement membrane in the primary bronchus and several medium-sized bronchi using Soft Imaging Systems software. Stomachs were retrieved and dissected longitudinally into equally sized pieces. For the quantitative assessment of *H. pylori* colonization, 1 stomach section was homogenized in Brucella broth, and serial dilutions were plated on horse blood plates for colony counting as described previously (18). For the quantitative assessment of gastric histopathology, Giemsa-stained paraffin-embedded stomach sections were scored on a scale from 0 to 6 for the parameters of chronic inflammation, atrophy, epithelial hyperplasia, and metaplasia, as described in detail previously (10). All gastric histopathology images were taken at ×100 or ×200 final magnification on a Leica Leitz DM RB microscope equipped with a DFC 420C camera. Images were acquired using Leica Application Suite 3.3.0 software.

Volunteers and assessment of gastritis and H. pylori colonization. Volunteers were recruited among blood donors at Sahlgrenska University Hospital after serologic analysis, and *H. pylori* infection was subsequently confirmed or excluded by culture on Scirrow plates. Gastric antrum biopsies were collected from 8 *H. pylori*–infected volunteers (7 males and 1 female, aged between 25–57 years) and 10 uninfected volunteers (5 males and 5 females, aged between 23–62 years) by endoscopy, immediately frozen in OCT medium, and later used for immunohistochemical staining of DCs. In addition, 1 biopsy from each volunteer was fixed in formalin, paraffin embedded, and examined by an experienced histopathologist for the grade of gastritis and the presence of *Helicobacter*-like organisms (HLOs) using the updated Sydney system (44). The serology, culture, and pathology results agreed in all cases, except those for 1 individual who did not have visible HLOs in the pathological examination but was culture and serology positive. Biopsy samples from 6 uninfected subjects were histologically normal without inflammation or HLOs, and biopsy samples from 4 uninfected subjects had mild chronic gastritis (score of 1) but no HLOs or active inflammation. In contrast, active chronic inflammation and HLOs were observed in biopsy samples from antrum of all *H. pylori*–infected subjects. The *H. pylori*–

infected individuals had a chronic inflammation score of 1.9 ± 0.4 (mean ± standard deviation), an active inflammation score of 1.4 ± 0.7, and an HLO score of 1.9 ± 0.8. Atrophy and metaplasia was seen in 1 of the *H. pylori*–infected subjects, and metaplasia alone was seen in 1 of the *H. pylori*–infected subjects.

Immunohistochemical and flow cytometric analysis of human gastric DCs. Antrum biopsies from *H. pylori*–infected and uninfected volunteers were cryosectioned into 8-μm thick sections, fixed for 10 minutes in ice-cold acetone, and endogenous peroxidase blocked with glucose-oxidase for 20 minutes. Sections were stained with mouse monoclonal antibodies to DC-SIGN (clone DCN46, BD Biosciences) in PBS containing 5% human and rabbit serum at room temperature for 30 minutes, followed by an HRP-conjugated rabbit antibody to mouse immunoglobulins (DakoCytomation). Isotype control antibodies were always run in parallel. The area stained with the respective antibodies was calculated relative to the total area of each tissue section, using Biopix image analysis software. For isolation of human gastric cells, 4 *H. pylori*–infected patients (2 males and 2 females, aged between 64–81 years) and 4 uninfected patients (1 male and 3 females, aged between 61–75 years) undergoing surgery for gastric adenocarcinoma ($n = 2$), pancreas cancer ($n = 5$), or chronic pancreatitis ($n = 1$) at Sahlgrenska University Hospital were also included in the study. Tissue was collected from antrum, and, in the gastric cancer patients, tissue was removed at least 5 cm away from the tumor. None of the patients had undergone radiotherapy or chemotherapy prior to the operation. *H. pylori* infection was determined based on serology, culture, and pathology reports; culture and serology agreed in 6 out of the 8 cases. Two seropositive cases with negative culture results, presumably due to extensive gastric atrophy and metaplasia, were deemed *H. pylori* positive. Antral tissue was stripped of muscle and connective tissue and cut into 5-mm pieces. Epithelial cells were removed by incubating the tissue for 15 minutes at 37°C with 2 mM EDTA and 1 mM dithiothreitol (Sigma-Aldrich) in Hank's balances salt solution supplemented with 2% FCS for a total of 3 times. Lamina propria leukocytes were then released by incubating the remaining tissue for 1 hour at 37°C with 100 U/ml collagenase type VIII (Sigma-Aldrich) and 0.1 mg/ml DNAse (Sigma-Aldrich) in RPMI 1640 containing 10% FCS. Isolated cells were stained with anti-CD80 (clone L307.4), CD86 (2331), CD83 (HB15e), DC-SIGN (DCN46), and HLA-DR (L243; all from BD Biosciences), and appropriate isotype controls were used. 7-Aminoactinomycin D (7AAD, Sigma-Aldrich) was used to exclude dead cells. Samples were acquired on an LSR-II flow cytometer (BD Biosciences) and analyzed using FlowJo software.

Preparation of murine BM-DCs and MLN-DCs and DC/T cell cocultures. For generation of BM-DCs, bone marrow isolated from the hind legs of wild-type, *Tlr2*$^{-/-}$, *Myd88*$^{-/-}$, or *cd11c*-hDC-SIGN transgenic donor mice was seeded at 50,000 cells per well in 96-well plates in RPMI/10% FCS and 4 ng/ml GM-CSF and cultured for 7 days. *E. coli* LPS (Serotype 0111:B4, Sigma-Aldrich) and the synthetic lipopeptide Pam3Cys-SKKKK (Genaxxon) were added at 0.5 μg/ml and 5 μg/ml final concentration, respectively, to induce BM-DC maturation. For the isolation of MLN-DCs, MLNs of individual mice were digested in 1 mg/ml collagenase (Sigma-Aldrich) for 30 minutes at 37°C, with shaking prior to filtering through a cell strainer (40 μm; BD Biosciences) and immunomagnetic isolation of DCs using mouse-specific CD11c microbeads (Miltenyi Biotec). DC cultures were infected o/n with wild-type *H. pylori* PMSS1 or an isogenic mutant lacking the *cagE* gene (ΔCagE) described previously (18). Bacteria were killed with 200 U penicillin/0.2 mg streptomycin/ml for 6 hours prior to the addition of T cells. $CD4^+CD25^-$ T cells were prepared from single cell suspensions of naive C57BL/6 spleens by immunomagnetic sorting (R&D Systems). DCs were cocultured with $CD4^+CD25^-$ T cells at a ratio of 1:2 (0.5×10^5 DCs to 1×10^5 T cells) in RPMI containing 10% FCS, 10 ng/ml recombinant TGF-β (rTGF-β) (PeproTech), 10 ng/ml recombinant IL-2 (rIL-2) (R&D Systems), and 1 μg/ml anti-CD3ε (BD Bioscience). After 72 hours of coculture, the cells were stained first for CD4 and CD25 and then, after fixation and permeabilization, for FoxP3 (FoxP3-APC, eBioscience). The percentage of $FoxP3^+$ $CD4^+$ T cells was assessed by FACS. IFN-γ secreted by cocultured DCs and T cells was quantified by ELISA (R&D Systems). IL12p40 and IL-10 (both from BD Biosciences) and IL-18 (MBL) production by DCs was assessed by ELISA. The quantification of IL-10 production by RT-PCR is described in the Supplemental Methods.

Preparation of murine gastric single cell suspensions and flow cytometry. One-sixth of every stomach (antrum and corpus) and corresponding MLNs were digested in 1 mg/ml collagenase for 30 minutes at 37°C, with shaking prior to mechanical disruption between glass slides and filtering through a cell strainer (40 μm). Single cell suspensions were stained directly for FACS analysis. The following antibodies were used: CD11c-biotin, CD4-FITC, CD4-APC, CD86-FITC, and CD40-APC (all from BD Biosciences) as well as CD45-PB and CD80-APC (both from BioLegend). IFN-γ-PE-Cy7 (BD) and FoxP3-APC (eBioscience) were used for intracellular staining. Prior to intracellular cytokine staining, cells were stimulated and blocked in medium containing 2.5 μg/ml Brefeldin A (AppliChem), 0.2 μM ionomycin (Santa Cruz Biotechnology Inc.), and 50 ng/ml phorbol 12-myristate 13-acetate (Sigma-Aldrich) for 5 hours; stained for extracellular markers; and fixed in 4% paraformaldehyde. Flow cytometry was performed on a Cyan ADP 9 instrument (Beckman Coulter) and analyzed using FlowJo software (TreeStar).

Statistics. GraphPad Prism (GraphPad Software) was used for statistical analyses. All *P* values were calculated by Mann-Whitney test unless otherwise indicated. In column bar graphs, the SEM is indicated by vertical bars. *P* values of less than 0.05 were considered statistically significant.

Study approval. All human studies were approved by the Regional Research Ethics Committee of Western Sweden, and informed consent was obtained from all participants. All animal experimentation was reviewed and approved by the Zurich Cantonal veterinary office (63/2008 and 170/2009 to A. Müller).

Acknowledgments

We thank all volunteers and patients for their participation in the study. This study was supported by the Swiss National Science Foundation and the Zurich University Research Priority Program in Systems Biology (to A. Müller), the Deutsche Forschungsgemeinschaft and Research Center for Immunology of the University of Mainz (to C. Taube), and the Swedish Science Council and Foundation for Strategic Research through the MIVAC centre of excellence (to M. Quiding-Järbrink). We are grateful to Burkhard Becher, Wolf-Dietrich Hardt, and Tim Sparwasser for providing mouse strains.

Received for publication September 15, 2011, and accepted in revised form December 14, 2011.

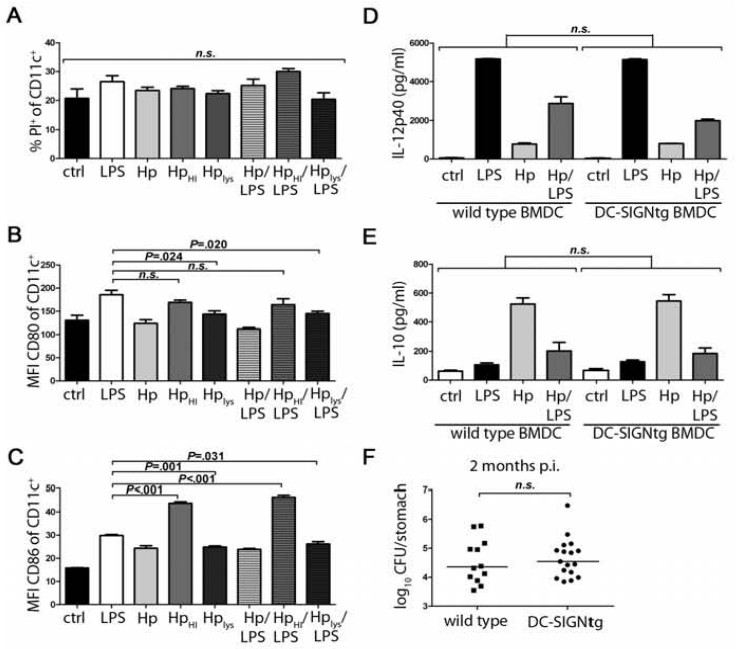
Address correspondence to: Anne Müller, Institute of Molecular Cancer Research, University of Zürich, Winterthurerstr. 190, 8057 Zürich, Switzerland. Phone: 41.44.635.3474; Fax: 41.44.635.3484; E-mail: mueller@imcr.uzh.ch.

1. Maldonado RA, von Andrian UH. How tolerogenic dendritic cells induce regulatory T cells. *Adv Immunol.* 2010;108:111–165.
2. Banchereau J, et al. Immunobiology of dendritic cells. *Annu Rev Immunol.* 2000;18:767–811.
3. Brodsky IE, Monack D. NLR-mediated control of inflammasome assembly in the host response against bacterial pathogens. *Semin Immunol.* 2009;21(4):199–207.
4. Ishii KJ, Koyama S, Nakagawa A, Coban C, Akira S. Host innate immune receptors and beyond: making sense of microbial infections. *Cell Host Microbe.*

2008;3(6):352–363.
5. Kretschmer K, Apostolou I, Hawiger D, Khazaie K, Nussenzweig MC, von Boehmer H. Inducing and expanding regulatory T cell populations by foreign antigen. *Nat Immunol.* 2005;6(12):1219–1227.
6. Marshall BJ, Warren JR. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet.* 1984;1(8390):1311–1315.
7. Parsonnet J, et al. Helicobacter pylori infection and the risk of gastric carcinoma. *N Engl J Med.* 1991;325(16):1127–1131.
8. Parsonnet J, et al. Helicobacter pylori infection and gastric lymphoma. *N Engl J Med.* 1994; 330(18):1267–1271.
9. Pritchard DM, Crabtree JE. Helicobacter pylori and gastric cancer. *Curr Opin Gastroenterol.* 2006; 22(6):620–625.
10. Sayi A, et al. The CD4+ T cell-mediated IFN-gamma response to Helicobacter infection is essential for clearance and determines gastric cancer risk. *J Immunol.* 2009;182(11):7085–7101.
11. Akhiani AA, et al. Protection against Helicobacter pylori infection following immunization is IL-12-dependent and mediated by Th1 cells. *J Immunol.* 2002;169(12):6977–6984.
12. Hitzler I, Oertli M, Becher B, Agger EM, Müller A. Dendritic cells prevent rather than promote immunity conferred by a helicobacter vaccine using a mycobacterial adjuvant. *Gastroenterology.* 2011;141(1):186–196.
13. Fox JG, Wang TC. Inflammation, atrophy, and gastric cancer. *J Clin Invest.* 2007;117(1):60–69.
14. Rieder G, Merchant JL, Haas R. Helicobacter pylori cag-type IV secretion system facilitates corpus colonization to induce precancerous conditions in Mongolian gerbils. *Gastroenterology.* 2005;128(5):1229–1242.
15. Correa P. A human model of gastric carcinogenesis. *Cancer Res.* 1988;48(13):3554–3560.
16. Toller IM, Altmeyer M, Kohler E, Hottiger MO, Muller A. Inhibition of ADP ribosylation prevents and cures helicobacter-induced gastric preneoplasia. *Cancer Res.* 2010;70(14):5912–5922.
17. Toller IM, Hitzler I, Sayi A, Mueller A. Prostaglandin E2 prevents Helicobacter-induced gastric preneoplasia and facilitates persistent infection in a mouse model. *Gastroenterology.* 2010;138(4):1455–1467.
18. Arnold IC, et al. Tolerance rather than immunity protects from Helicobacter pylori-induced gastric preneoplasia. *Gastroenterology.* 2011;140(1):199–209.
19. Blaser MJ, Chen Y, Reibman J. Does Helicobacter pylori protect against asthma and allergy? *Gut.* 2008;57(5):561–567.
20. Chen Y, Blaser MJ. Inverse associations of Helicobacter pylori with asthma and allergy. *Arch Intern Med.* 2007;167(8):821–827.
21. Chen Y, Blaser MJ. Helicobacter pylori colonization is inversely associated with childhood asthma. *J Infect Dis.* 2008;198(4):553–560.
22. Reibman J, et al. Asthma is inversely associated with Helicobacter pylori status in an urban population. *PLoS One.* 2008;3(12):e4060.
23. Arnold IC, et al. Helicobacter pylori infection prevents allergic asthma in mouse models through the induction of regulatory T cells. *J Clin Invest.* 2011;121(8):3088–3093.
24. Gringhuis SI, den Dunnen J, Litjens M, van der Vlist M, Geijtenbeek TB. Carbohydrate-specific signaling through the DC-SIGN signalosome tailors immunity to Mycobacterium tuberculosis, HIV-1 and Helicobacter pylori. *Nat Immunol.* 2009;10(10):1081–1088.
25. Schaefer M, et al. Decreased pathology and prolonged survival of human DC-SIGN transgenic mice during mycobacterial infection. *J Immunol.* 2008;180(10):6836–6845.
26. Rad R, et al. Extracellular and intracellular pattern recognition receptors cooperate in the recognition of Helicobacter pylori. *Gastroenterology.* 2009;136(7):2247–2257.
27. Sayi A, et al. TLR-2-activated B cells suppress Helicobacter-induced preneoplastic gastric immunopathology by inducing T regulatory-1 cells. *J Immunol.* 2011;186(2):878–890.
28. Kiriya K, et al. Essential role of Peyer's patches in the development of Helicobacter-induced gastritis. *Int Immunol.* 2007;19(4):435–446.
29. Nagai S, et al. Role of Peyer's patches in the induction of Helicobacter pylori-induced gastritis. *Proc Natl Acad Sci U S A.* 2007;104(21):8971–8976.
30. Geijtenbeek TB, et al. Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. *Cell.* 2000;100(5):575–585.
31. Weyermann M, Rothenbacher D, Brenner H. Acquisition of Helicobacter pylori infection in early childhood: independent contributions of infected mothers, fathers, and siblings. *Am J Gastroenterol.* 2009;104(1):182–189.
32. Li W, et al. Helicobacter pylori infection is a potential protective factor against conventional multiple sclerosis in the Japanese population. *J Neuroimmunol.* 2007;184(1–2):227–231.
33. Luther J, Dave M, Higgins PD, Kao JY. Association between Helicobacter pylori infection and inflammatory bowel disease: a meta-analysis and systematic review of the literature. *Inflamm Bowel Dis.* 2010;16(6):1077–1084.
34. Sladek M, Jedynak-Wasowicz U, Wedrychowicz A, Kowalska-Duplaga K, Pieczarkowski S, Fyderek K. [The low prevalence of Helicobacter pylori gastritis in newly diagnosed inflammatory bowel disease children and adolescent]. *Przegl Lek.* 2007;64 suppl 3:65–67.
35. Robinson K, et al. Helicobacter pylori-induced peptic ulcer disease is associated with inadequate regulatory T cell responses. *Gut.* 2008;57(10):1375–1385.
36. Gewirtz AT, Yu Y, Krishna US, Israel DA, Lyons SL, Peek RM Jr. Helicobacter pylori flagellin evades toll-like receptor 5-mediated innate immunity. *J Infect Dis.* 2004;189(10):1914–1920.
37. Moran AP. Structure and conserved characteristics of Campylobacter jejuni lipopolysaccharides. *J Infect Dis.* 1997;176 suppl 2:S115–S121.
38. Moran AP, Lindner B, Walsh EJ. Structural characterization of the lipid A component of Helicobacter pylori rough- and smooth-form lipopolysaccharides. *J Bacteriol.* 1997;179(20):6453–6463.
39. Zaki MH, Boyd KL, Vogel P, Kastan MB, Lamkanfi M, Kanneganti T-D. The NLRP3 Inflammasome Protects against Loss of Epithelial Integrity and Mortality during Experimental Colitis. *Immunity.* 2010;32(3):379–391.
40. Dupaul-Chicoine J, et al. Control of intestinal homeostasis, colitis, and colitis-associated colorectal cancer by the inflammatory caspases. *Immunity.* 2010;32(3):367–378.
41. Onishi Y, Fehervari Z, Yamaguchi T, Sakaguchi S. Foxp3+ natural regulatory T cells preferentially form aggregates on dendritic cells in vitro and actively inhibit their maturation. *Proc Natl Acad Sci U S A.* 2008;105(29):10113–10118.
42. Reuter S, et al. Mast cell-derived tumour necrosis factor is essential for allergic airway disease. *Eur Respir J.* 2008;31(4):773–782.
43. Bopp T, et al. Inhibition of cAMP degradation improves regulatory T cell-mediated suppression. *J Immunol.* 2009;182(7):4017–4024.
44. Dixon MF, Genta RM, Yardley JH, Correa P. Classification and grading of gastritis. The updated Sydney System. International Workshop on the Histopathology of Gastritis, Houston 1994. *Am J Surg Pathol.* 1996;20(10):1161–1181.

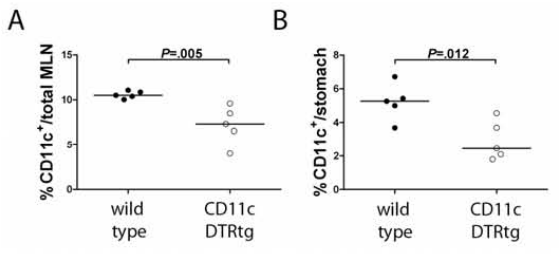
Supplemental Figures:

Supplemental Figure 1



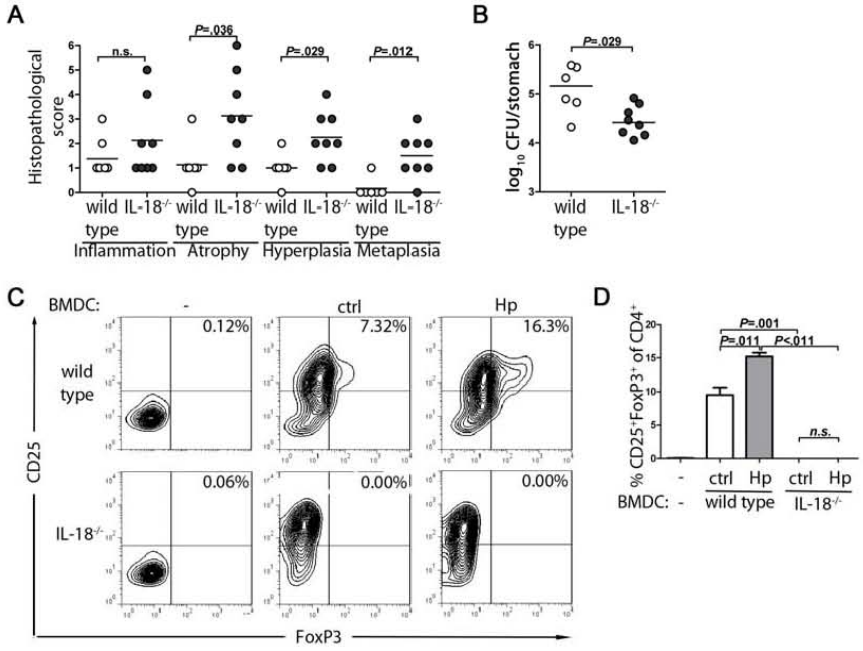
Suppl. Figure 1. BM-DC infection with *H. pylori* does not induce cytotoxicity and treatment of BM-DCs with *H. pylori* sonicate, but not heat-inactivated bacteria, phenocopies the effect of live infection on the LPS-mediated up-regulation of CD80 and CD86; DC-SIGN is not required for the inhibitory activity of *H. pylori*. (**A-C**) BM-DCs were infected with *H. pylori* strain PMSS1 at a multiplicity of infection (MOI) of 50 and/or treated with 0.5μg/ml *E. coli* LPS for 16h prior to the flow cytometric analysis of propidium iodide-positive cells **(A)**, CD80 **(B)** and CD86 expression **(C)**. Additional wells were treated with 10μg/ml *H. pylori* lysate (obtained by sonication, "lys") or heat-inactivated bacteria ("HI") corresponding to an MOI of 50 during the entire 16h exposure to *E. coli* LPS where indicated. **(D-F)** Transgenic expression of human DC-SIGN (DC-SIGNtg) under the *cd11c* promoter does not affect the inhibitory activity of *H. pylori* infection on LPS-induced DC maturation, as assessed by IL-12 and IL-10 ELISA, and *in vivo* infection experiments. Wild type and DC-SIGN-transgenic BM-DCs were infected with *H. pylori* strain PMSS1 at a multiplicity of infection (MOI) of 50 and/or treated with 0.5μg/ml *E. coli* LPS for 16h prior to the assessment of IL-12 and IL-10 production by ELISA **(D,E)**. **(F)** Wild type and DC-SIGN-transgenic mice were infected with *H. pylori* strain PMSS1 for two months and analyzed with respect to *H. pylori* colonization levels by plating and colony counting of gastric homogenate.

Supplemental Figure 2



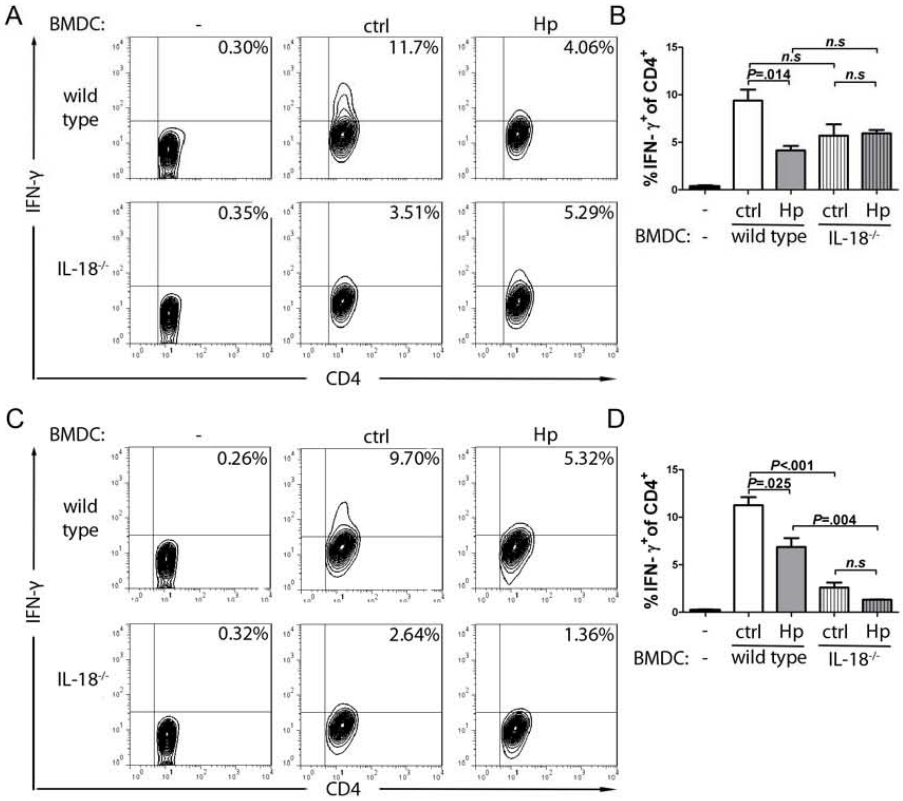
Suppl. Figure 2. DC numbers in wild type and CD11c-DTR transgenic mice after two weeks of DT administration. (**A**) % $CD11c^+$ cells in the MLN. (**B**) % $CD11c^+$ cells in the stomach.

Supplemental Figure 3



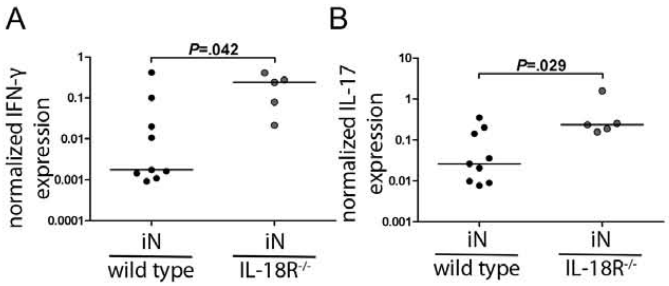
Suppl. Figure 3. IL-18 is required for the restriction of gastric *H. pylori* infection-induced immunopathology and for ovalbumin-specific Treg induction by *H. pylori*-exposed DCs. (A) Histopathology scores of wild type and IL-18$^{-/-}$ mice infected at six weeks of age with *H. pylori* PMSS1 for 1 month; horizontal lines indicate the means. Scores on a scale from 0-6 were assigned independently for the parameters gastric inflammation, atrophy, epithelial hyperplasia and intestinal metaplasia, as described in detail previously (1, 2) **(B)** *H. pylori* PMSS1 colonization levels as assessed by colony count assay; medians are represented by horizontal lines. (**C,D**) Wild type and IL-18$^{-/-}$ BM-DCs treated as described in Figure 7 were loaded with 20µg/ml ovalbumin prior to co-culturing with OTII T-cells in the presence of rTGF-β and rIL-2. CD25 and FoxP3 staining of the CD4^{+} gate are shown for representative mice (**D**) along with the quantification for all mice (**D**).

Supplemental Figure 4



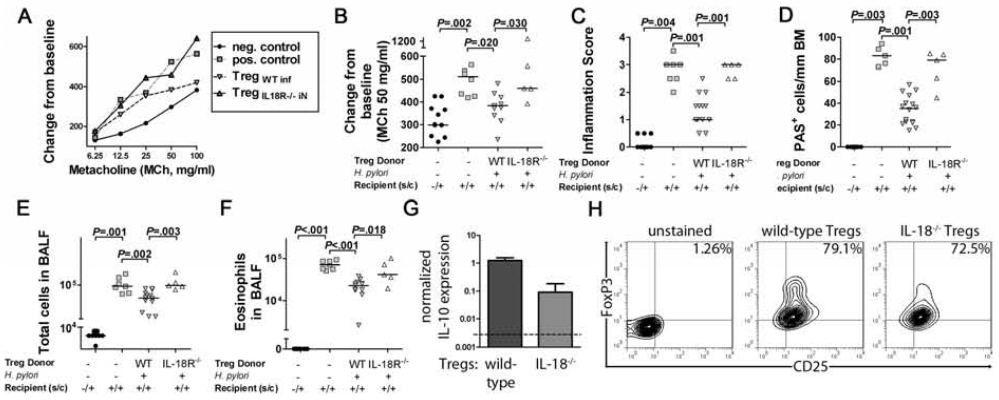
Suppl. Figure 4. IL-18$^{-/-}$ BM-DCs fail to induce IFN-γ expression in co-cultured T-cells. (**A,B**) Wild type and IL-18$^{-/-}$ BM-DCs were infected as described in Figure 2A prior to co-culturing with immunomagnetically isolated, splenic OTII CD4^{+}CD25^{-} T-cells for 3 days in the presence of 10ng/ml rIL-2 and 1µg/ml anti-CD3ε mAb. (**C,D**) The same cells were alternatively loaded with 20µg/ml ovalbumin during the 16h *H. pylori* infection; under these circumstances, anti-CD3ε mAb was not included in the BM-DC/T-cell co-cultures. IFN-γ-producing CD4^{+} T-cells were quantified by intracellular cytokine staining; representative FACS plots and averages of triplicate measurements +/- SEM are shown in A and C, and B and D, respectively. T-cells cultured without DCs served as controls (-).

Supplemental Figure 5



Suppl. Figure 5. Treg differentiation and the development of *H. pylori*-specific tolerance requires IL-18 signaling *in vivo*. (**A,B**) Gastric mucosal IFN-γ and IL-17 expression of the C57BL/6 wild type and BL/6.IL-18R$^{-/-}$ mice neonatally infected with *H. pylori* shown in Figure 8C-F, as determined by qPCR and normalized to GAPDH expression.

Supplemental Figure 6



Suppl. Figure 6. IL-18 signaling is required for the generation of Tregs with suppressive activity and for T-regulatory IL-10 production (**A-F**) Wild type C57BL/6 mice were sensitized with two i.p. doses of alum-adjuvanted ovalbumin prior to challenge with aerosolized ovalbumin 2 weeks after the last sensitization. Two groups of sensitized recipients received 250,000 immunomagnetically isolated $CD4^+CD25^+$ T-cells isolated from the MLNs of either neonatally infected wild type or IL-$18R^{-/-}$ donors one day before the first challenge. Negative controls were challenged without prior sensitization. (**A,B**) Airway hyper-responsiveness as assessed by challenge with increasing doses of metacholine (**A**) and the 50 mg/ml dose (**B**), respectively. (**C,D**) Tissue inflammation and goblet cell metaplasia as assessed and scored on H&E and PAS-stained tissue sections. (**E**) Total cells contained in 1ml of BALF. (**F**) Eosinophils in 1ml of BALF. Horizontal lines indicate medians; "s/c" stands for "sensitized/challenged". (**G**) $CD4^+CD25^+$ T-cells were immunomagnetically isolated from single cell MLN suspensions of neonatally infected (PMSS1, four weeks of infection) C57BL/6 wild type or BL/6.IL-$18^{-/-}$ mice (five per group) and subjected to RNA isolation and IL-10-specific real time RT-PCR. IL-10 transcript levels were normalized to GAPDH expression. Data represent means +/- SEM. The dashed line indicates the average IL-10 expression of Tregs from three uninfected wild type and three uninfected IL-$18^{-/-}$ controls. (**H**) Representative FACS plots of FoxP3 and CD25 staining demonstrating that $CD4^+CD25^+$ T-cells from IL-$18^{-/-}$ donors do not differ from wild type $CD4^+CD25^+$ T-cells in terms of FoxP3 expression.

Supplemental References:

1. Arnold, I.C., Lee, J.Y., Amieva, M.R., Roers, A., Flavell, R.A., Sparwasser, T., and Muller, A. 2011. Tolerance rather than immunity protects from Helicobacter pylori-induced gastric preneoplasia. *Gastroenterology* 140:199-209.
2. Sayi, A., Kohler, E., Hitzler, I., Arnold, I., Schwendener, R., Rehrauer, H., and Muller, A. 2009. The CD4+ T cell-mediated IFN-gamma response to Helicobacter infection is essential for clearance and determines gastric cancer risk. *J Immunol* 182:7085-7101.

Supplemental Methods:

Real time RT-PCR

For real-time RT-PCR, total RNA was isolated from one-sixth of every stomach (antrum and corpus) using NucleoSpin RNA II kits (Macherey-Nagel). The corresponding cDNA served as a template for real-time PCR performed using the LightCycler 480 SYBR Green I master kit (Roche). Absolute values of IFN-γ, IL-17 and IL-10 expression were normalized to GAPDH expression (conditions: Tm 55°C, 50 cycles; primers: GAPDH fw GAC ATT GTT GCC ATC AAC GAC C; GAPDH rv CCC GTT GAT GAC CAG CTT CC; IFN-γ fw CAT GGC TGT TTC TGG CTG TTA CTG; IFN-γ rv GTT GCT GAT GGC CTG ATT GTC TTT; IL-17 fw GCT CCA GAA GGC CCT CAG A; IL-17 rv AGC TTT CCC TCC GCA TTG A; IL-10 fw CTA GAG CTG CGG ACT GCC TTC A; IL-10 rv CCT GCT CCA CTG CCT TGC TCT TAT).

3.2 DENDRITIC CELLS PREVENT RATHER THAN PROMOTE IMMUNITY CONFERRED BY *HELICOBACTER* VACCINE USING A MYCOBACTERIAL ADJUVANT

Article published in Gastroenterology, 2011

Authors: Iris Hitzler, Mathias Oertli, Burkhard Becher, Else-Marie Agger and Anne Müller

Contribution: IH designed and conducted most of the experiments and wrote the manuscript together with AM. I contributed figure 6E-F. BB and EMA provided helpful tools

GASTROENTEROLOGY 2011;141:186–196

Dendritic Cells Prevent Rather Than Promote Immunity Conferred by a *Helicobacter* Vaccine Using a Mycobacterial Adjuvant

IRIS HITZLER,* MATHIAS OERTLI,* BURKHARD BECHER,‡ ELSE MARIE AGGER,§ and ANNE MÜLLER*

*Institute of Molecular Cancer Research and ‡Institute of Experimental Immunology, University of Zurich, Zurich, Switzerland; §Department of Infectious Disease Immunology, Statens Serum Institut, Copenhagen, Denmark

BACKGROUND & AIMS: Immunization against the gastric bacterium *Helicobacter pylori* could prevent many gastric cancers and other disorders. Most vaccination protocols used in preclinical models are not suitable for humans. New adjuvants and a better understanding of the correlates and requirements for vaccine-induced protection are needed to accelerate development of vaccines for *H pylori*. **METHODS:** Vaccine-induced protection against *H pylori* infection and its local and systemic immunological correlates were assessed in animal models, using cholera toxin or CAF01 as adjuvants. The contribution of B cells, T-helper (Th)–cell subsets, and dendritic cells to *H pylori*–specific protection were analyzed in mice. **RESULTS:** Parenteral administration of a whole-cell sonicate, combined with the mycobacterial cell-wall–derived adjuvant CAF01, protected against infection with *H pylori* and required cell-mediated, but not humoral, immunity. The vaccine-induced control of *H pylori* was accompanied by Th1 and Th17 responses in the gastric mucosa and in the gut-draining mesenteric lymph nodes; both Th subsets were required for protective immunity against *H pylori*. The numbers of memory CD4+ T cells and neutrophils in gastric tissue were identified as the best correlates of protection. Systemic depletion of dendritic cells or regulatory T cells during challenge infection significantly increased protection by overriding immunological tolerance mechanisms activated by live *H pylori*. **CONCLUSIONS: Parenteral immunization with a *Helicobacter* vaccine using a novel mycobacterial adjuvant induces protective immunity against *H pylori* that is mediated by Th1 and Th17 cells. Tolerance mechanisms mediated by dendritic cells and regulatory T cells impair *H pylori* clearance and must be overcome to improve immunity.**

Keywords: Gastric Cancer; Bacteria; Ulcer; Gastric Adenocarcinoma.

BASIC AND TRANSLATIONAL AT

Helicobacter pylori colonizes half of the world's population and chronic gastric infection with this bacterium leads to the development of gastric ulcers, gastric adenocarcinoma, or mucosa-associated lymphoid tissue lymphoma in 1%–10% of infected individuals.[1] The standard *H pylori* eradication therapy consists of 2 to 3 antibiotics and an acid suppressant; however, antibiotic resistance rates are rising,[2] a subset of individuals relapse even after efficient eradication,[3] and the infection is often not diagnosed until gastric premalignant lesions have progressed to an irreversible stage.[4] Immunization against *H pylori*, therefore, represents an attractive alternative strategy for the prevention of gastric cancer and other *Helicobacter*-associated gastric disorders.

No vaccine regimens are currently available for human use. The gold standard vaccination protocol that has been used extensively in preclinical models combines the mucosal adjuvant cholera toxin (CT) with whole-cell preparations of *H pylori* or *Helicobacter felis* into orally administered formulations.[5–8] Because of the toxicity of CT, human trials have bypassed this effective adjuvant in favor of other less toxic compounds, such as the heat-labile enterotoxin of *Escherichia coli*, which was combined with recombinant urease B- or whole-cell preparations to generate well-tolerated, but largely ineffective formulations.[9–11] A live recombinant *Salmonella enterica* serovar Typhi Ty21a vaccine expressing *H pylori*'s urease A and B subunits also did not show sufficient immunogenicity in human volunteers,[12,13] but confirmed a direct correlation between *H pylori*–specific T-cell responses and bacterial clearance.[14]

Protective immunity against *H pylori* can be achieved by either Th1 and/or Th17-polarizing adjuvants[5,15–18] or by aluminum-based adjuvants that stimulate Th2-polarized responses.[19,20] Both complete Freund's adjuvant and alum have been used successfully in preventive[20,21] and even therapeutic[19] vaccination strategies. An alum-adjuvanted parenteral vaccine using the *H pylori* antigens VacA, CagA, and NAP was recently reported to be immunogenic and safe in humans.[22] Here we focus on a novel adjuvant for Th1/Th17 vaccination that incorporates a synthetic analog (trehalose-6,6-dibehenate) of the mycobacterial cell wall glycolipid trehalose-6,6-dimycolate into a cationic liposome-based adjuvant formulation (CAF01).[23] CAF01 facilitates antigen uptake and presentation by dendritic cells (DCs) and macrophages[24,25] and confers protective immunity to a variety of infectious agents, including *Mycobacterium tuberculosis*, *Chlamydia muridarum*, and the malarial parasite *Plasmodium yoelii*.[26,27] The mixed Th1/Th17

Abbreviations used in this paper: APC, allophycocyanin; CT, cholera toxin; DC, dendritic cell; DT, diphtheria toxin; IFN-γ, interferon-γ; IL, interleukin; IP, intraperitoneal; MHC, major histocompatibility complex; MLN, mesenteric lymph nodes; PB, pacific blue; SC, subcutaneous; Th1/2/17, T-helper type 1/2/17; Treg, regulatory T cell.

0016-5085/$36.00
doi:10.1053/j.gastro.2011.04.009

T-cell responses elicited by CAF01-adjuvanted vaccination have been particularly well-characterized in the context of *M tuberculosis* infection.[23]

Here, we compared various routes of administration of a CAF01-adjuvanted *H pylori* whole-cell vaccine to a CT-adjuvanted immunization protocol with respect to protection from autologous challenge infection. We show that parenteral—but not mucosal—immunization with CAF01 elicits protective immunity to *H pylori* challenge infection that is antibody-independent, but requires both Th1 and Th17 subsets. Other correlates of protection included gastric memory T-cell, neutrophil, and mast cell infiltration. We further report here that immunoregulatory mechanisms involving DCs and regulatory T cells (Treg) impair protective immunity; consequently, the systemic depletion of DCs or Treg during challenge infection significantly improved the efficacy of *H pylori*–specific vaccination.

Materials and Methods

Animal Experimentation

C57BL/6 wild-type, major histocompatibility complex (MHC) II$^{-/-}$, JHT$^{-/-}$ (which lack J_H segments and the intron enhancer in the IgH locus and therefore cannot generate B cells),[28] interleukin (IL)-12 p35$^{-/-}$, IL-12/IL-23 p40$^{-/-}$, and *CD11c*-DTR tg mice (expressing the diphtheria toxin [DT] receptor under the control of the *CD11c* promoter) were originally purchased from Charles River Laboratories (Wilmington, MA). *FoxP3*-EGFP-DTR tg mice[29] and IL-23 p19$^{-/-}$ mice were kindly provided by Tim Sparwasser and Regeneron Pharmaceuticals, respectively. All mice were bred at a University of Zurich specific pathogen-free facility. Mixed-sex groups were included in studies at 6 weeks of age. All animal experimentation was reviewed and approved by the Zurich cantonal veterinary office. Mice were immunized 3 times at weekly intervals. A stable CAF01 formulation consisting of dimethyldioctadecylammonium bromide and a,a′-trehalose 6,6′-dibehenate (Avanti Polar Lipids, Alabaster, AL) was prepared by the lipid film hydration method, as described previously.[26] For CAF01 vaccinations, 250 μg *H pylori* SS1[30] whole-cell sonicate was given per dose. In the case of intraperitoneal (IP), oral, or subcutaneous (SC) immunization at the base of the neck, the total volume was 200 μL. For intranasal immunizations, a total volume of 50 μL was given in drops of 12.5 μL to alternating nostrils of mice anesthetized with isoflurane (Minrad, Buffalo, NY). For CT immunization, mice were administered 500 μg sonicate along with 10 μg CT (List Biologicals, Campbell, CA) by oral gavage. Two weeks after the last immunization, immunized and naïve mice were infected with 10^8 *H pylori* SS1 (colony-forming unit estimated based on optical density) grown as described previously.[7] Mice were sacrificed 2 weeks after challenge. Bacterial colonization was assessed by colony count assay as described earlier.[7] DT (Sigma-Aldrich, St Louis, MO) was injected IP every 2 days for the depletion of DCs (4 ng/g body weight) or of Treg (40 ng/g body weight).

Preparation of Gastric and Mesenteric Lymph Node Single-Cell Suspensions, Flow Cytometry, Enzyme-Linked Immunosorbent Assay, Quantitative Reverse Transcription Polymerase Chain Reaction, and Treg Conversion Assay

One-sixth of every stomach (antrum and corpus) and corresponding mesenteric lymph nodes were digested in 1 mg/mL collagenase (Sigma-Aldrich) for 45 minutes at 37°C with shaking before mechanical disruption between glass slides and filtering. Single-cell suspensions were either stained directly for flow cytometric analysis or seeded at 200-k cells/well into 96-well round-bottom plates. The following antibodies were used: CD4–fluorescein isothiocyanate, CD4-allophycocyanin (APC) (BD Biosciences, San Diego, CA), CD45-pacific blue (PB), Ly6G-APC, CD62L-APC, CD44-PB, rat anti-mouse CD117 (all BioLegend, San Diego, CA), followed by goat anti-rat fluorescein isothiocyanate (Sigma-Aldrich) and CD11c-biotin (BD Biosciences), followed by streptavidin-PB (Life Technologies, Carlsbad, CA). Interferon-γ (IFN-γ)-phycoerythrin-Cy7 (BD), IL-17–APC, and FoxP3-APC (all eBioscience, San Diego, CA) were used for intracellular staining. Before intracellular cytokine staining, cells were stimulated and blocked in 2.5 μg/mL Brefeldin A (AppliChem, Darmstadt, Germany), 0.2 μM ionomycin (Santa Cruz Biotechnology, Santa Cruz, CA), and 50 ng/mL phorbol 12-myristate 13-acetate (Sigma-Aldrich) for 5 hours, stained for extracellular markers, and fixed in 4% paraformaldehyde. Flow cytometry was performed on a Cyan ADP 9 instrument (Beckman Coulter, Brea, CA) and analyzed using FlowJo software (TreeStar, Ashland, OR). Enzyme-linked immunosorbent assay and quantitative reverse transcription polymerase chain reaction techniques, as well as the protocols for Treg conversion are described in the Supplementary Methods.

Statistics

GraphPad Prism (GraphPad Software, La Jolla, CA) was used for statistical analyses. Colonization counts were compared by Mann–Whitney test. All other indicated *P* values were calculated by Student *t* test.

Results

Parenteral CAF01-Adjuvanted Vaccination Confers Protective Immunity to H pylori

To test the efficacy of the synthetic mycobacterial adjuvant CAF01 in *H pylori*–specific vaccination, we immunized mice with either the gold standard CT-adjuvanted, orally administered *H pylori* sonicate vaccine, or mixed the same sonicate with CAF01 for SC, IP, intranasal, and oral administration. Two weeks after the last of 3 weekly doses, all immunized mice as well as nonimmunized controls were challenged with 10^8 live *H pylori* SS1 and sacrificed 2 weeks later (see timetable in Figure 1*A*).

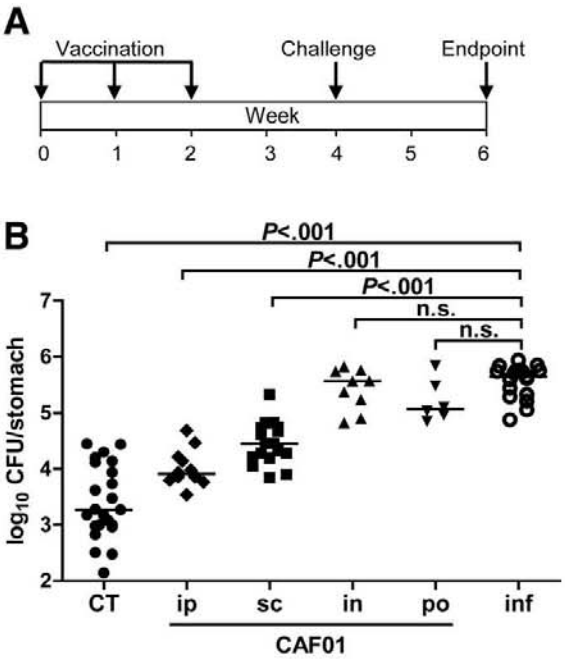


Figure 1. CAF01 adjuvant elicits protective immunity against *H pylori*. (*A*) Timetable of CAF01 and CT immunizations. (*B*) *H pylori* colonization levels as assessed by colony count assay (colony-forming units) for mice vaccinated orally with CT or with CAF01 adjuvant via different routes (ip, intraperitoneal; sc, subcutaneous; in, intranasal; po, oral) as well as for infected controls (inf). *Horizontal lines* represent median values. Combined data from 2 to 4 independent studies are shown.

The CAF01-adjuvanted vaccine provided protective immunity when administered parenterally (SC or IP), but not when given via mucosal routes (ie, intranasal or oral; Figure 1*B*). Speculating that the challenge dose might influence protection, we infected groups of SC CAF01-immunized mice with challenge doses ranging from 10^3 to 10^9 *H pylori*. Interestingly, all challenged mice were protected to the same degree, arguing that even minute *H pylori* burdens cannot be cleared completely by CAF01-vaccinated hosts (Supplementary Figure 1*A*). Assessing vaccine efficacy at a later time point post challenge (5 weeks) did not affect the vaccination outcome (Supplementary Figure 1*B*). The combined results suggest that the novel adjuvant CAF01 shows efficacy in *H pylori*–specific systemic vaccination strategies.

Th1-Mediated Immunity Is Associated With CAF01-Induced Protection Against H pylori

In order to identify immunological correlates of CAF01 vaccine-mediated protective immunity to *H pylori*, we examined *H pylori*–specific serum and mucosal antibody titers in the treatment arms outlined here. Most immunization strategies (except oral vaccination with CAF01) induced significantly elevated levels of *H pylori*–specific serum and mucosal IgG but not IgA; the mucosal IgG responses were generally more predictive of vaccination outcome than the serum IgG responses (Figure 2*A–C*). As relative IgG2c and IgG1 levels serve as indicators of systemic Th1 and Th2 polarization of $CD4^+$ Th cells, we separately quantified both IgG subclasses and calculated their ratios. Interestingly, only the IP- or SC-immunized, protected mice produced significant amounts of serum and mucosal IgG2c, whereas IgG1 was also generated by unprotected mice (Figure 2*D, E*), indicating that the Th1 polarization of activated T cells is a correlate and possible prerequisite for protection. To examine this further, we compared gastric levels of the Th1 and Th17 signature cytokines IFN-γ and IL-17 in the various treatment arms. Indeed, mice that were well-protected, ie, the CT- as well as the IP and SC CAF-immunized groups showed a significantly higher level of IFN-γ and IL-17 expression at the site of challenge infection than unprotected mice or infected-only controls (Figure 2*F*). Overall, these data imply that indicators of Th1/Th17 polarization detected either locally or systemically represent useful correlates of protective immunity to *H pylori*.

Antibodies Are Dispensable and $CD4^+$ T Cells Are Required for CAF01-Mediated Protective Immunity

Previous studies have shown that *H pylori*–specific antibodies may be generated in vaccinated/challenged animals, but are not necessarily required for protection.[31–33] In order to clarify this issue in the context of our vaccination strategies, we immunized $JHT^{-/-}$ mice lacking functional B cells with either the CT- or the CAF01-adjuvanted vaccine (Figure 3*A*, *left* and *right panels*). Both groups of vaccinated mice controlled the challenge infection as well as wild-type animals (Figure 3*A*), indicating that neither B cells nor *H pylori*–specific antibodies are required for protection. In contrast, mice lacking a functional $CD4^+$ T-cell compartment due to a targeted deletion of the MHC class II locus were incapable of controlling the challenge infection in both immunization arms of the study (Figure 3*B*). Therefore, B cells are dispensable and MHC class II–restricted T cells are strictly required for *H pylori*–specific immunity in these models.

Immune Cell Infiltration Into the Stomach Correlates With the Level of Protection

To identify additional correlates and a possible mechanism of vaccine-induced protection, we characterized the immune cell populations infiltrating the gastric mucosa in a panel of CT- or CAF01-immunized mice compared to control-infected mice. The protection levels achieved by the various treatment arms were representative in this study (Figure 4*A*). Whole-stomach, single-cell preparations were generated for individual mice and analyzed flow cytometrically with respect to the infiltration of $CD45^+$ leukocytes (Figure 4*B*), $CD4^+$ T cells (Figure 4*C*), and $CD4^+CD62L^-CD44^+$ activated memory T cells (Figure 4*D*). All 3 populations were elevated in those treatment arms that had successfully controlled the challenge infection, but not in the unprotected mice (Figure

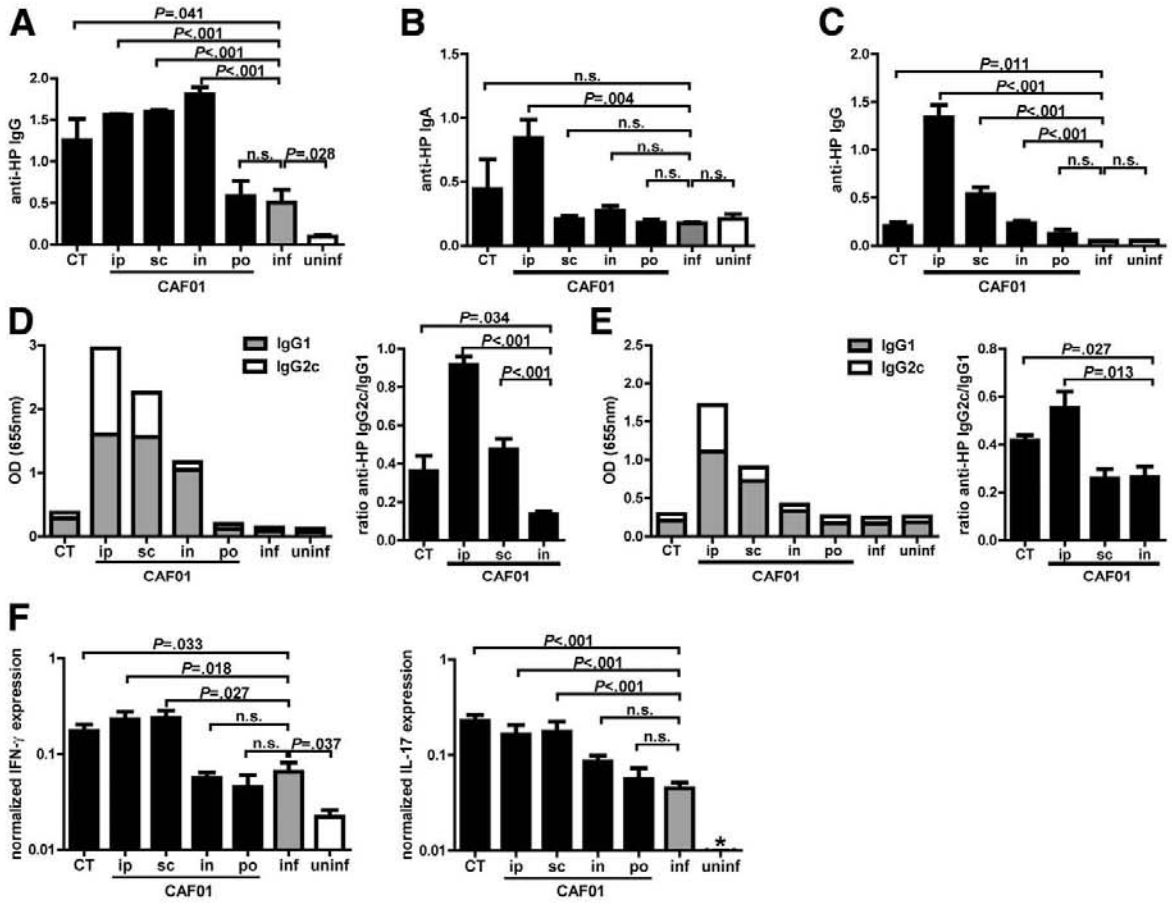


Figure 2. Protection against *H pylori* (HP) requires Th1-biased immunity. (*A–C*) Relative levels of *H pylori*–specific serum IgG (*A*), mucosal IgA (*B*), and mucosal IgG (*C*) in immunized, infected, and uninfected mice, measured by enzyme-linked immunosorbent assay and represented as optical density values. (*D*) Serum and (*E*) mucosal *H pylori*–specific IgG1 and IgG2c levels of the same mice (*left panels*), as well as IgG2c/IgG1 ratios (*right panels*) for mice with antibody levels above baseline. (*F*) Gastric IFN-γ and IL-17 transcript levels as determined by real-time reverse transcription polymerase chain reaction, normalized to glyceraldehyde-3-phosphate dehydrogenase expression. Groups of 4–10 animals were examined in *panels A–F*; data are shown as mean ± SEM. *Not detectable.

4*B–D*). In line with previously published data implicating Ly6-G$^+$ neutrophils and c-Kit$^+$ mast cells in the clearance of *H pylori*,[5,34] both populations were enriched in the mucosa of protected but not unprotected mice (Figure 4*E, F*). Overall, these data suggest that the gastric infiltration of immune cells, in particular of memory T cells, leukocytes, and neutrophils (and to a lesser extent of mast cells), serves as a reliable marker of protection against *H pylori* infection and highlights the importance of local mucosal immune responses in the control of this extracellular, luminal pathogen.

CAF01-Adjuvanted Vaccination Elicits a Mixed Th1/Th17 Response Upon Challenge Infection; Both Th Subsets Are Required for Protective Immunity Against H pylori

Th1 cells as well as Th17 cells have been implicated in the control of *H pylori* infection after CT-adjuvanted vaccination[5,18] and CAF01 is known to induce mixed Th1 and Th17 responses.[23] Therefore, we characterized the cytokine expression profiles of CD4$^+$ T cells infiltrating the stomachs of CAF01-immunized mice after challenge infection. Intracellular cytokine staining for IFN-γ and IL-17 revealed that the numbers of both stomach-infiltrating IFN-γ$^+$ CD4$^+$ T cells and IL-17$^+$ CD4$^+$ T cells were significantly higher in CAF01-immunized, challenged mice than in infected-only controls; infected mice in turn exhibited higher numbers of cytokine-positive cells than uninfected controls (Figure 5*A*). Having shown earlier that the gut-draining mesenteric lymph nodes (MLN) are important sites of *H pylori*–specific T-cell priming,[35] we hypothesized that similar differences in IFN-γ– and IL-17–producing T cells should be detectable in single-cell MLN preparations. We cultured MLN suspensions of individual mice for 4 days before restimulation and intracellular cytokine staining for IFN-γ and IL-17. Again, significantly more cytokine-expressing T cells of both

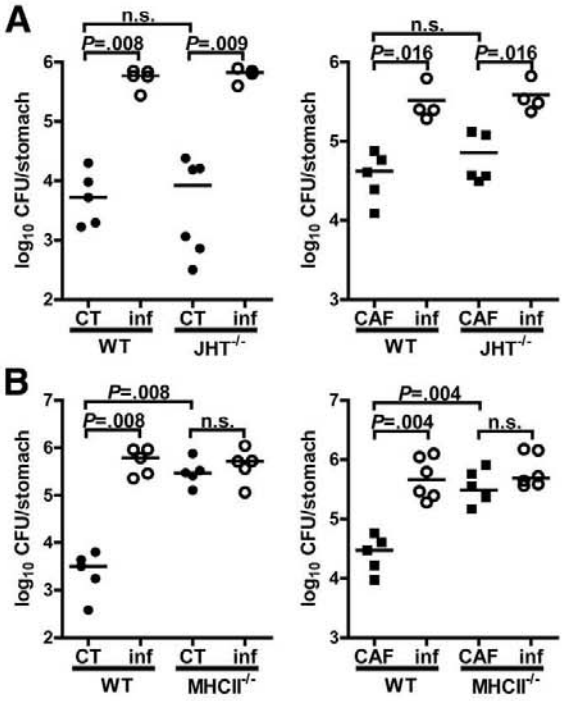


Figure 3. T cells but not B cells are required for CAF01-induced protection. (*A–B*) Colonization levels of (*A*) JHT$^{-/-}$ and (*B*) MHC class II$^{-/-}$ mice that were vaccinated with either CT (*left panels*) or CAF01 SC (*right panels*) or remained unvaccinated (inf) in comparison to wild-type mice.

populations were detected in the CAF01-vaccinated compared to infected-only mice, which in turn exhibited higher numbers than uninfected controls (Figure 5*B*). Further experiments confirmed that IFN-γ and IL-17 produced in the MLN of vaccinated mice were indeed derived from *H pylori*–specific T cells (Supplementary Figure 2). The combined observations suggest that mixed Th1/Th17 responses are a general hallmark of protective immunity to *H pylori*. To clarify whether both Th subsets are essential for vaccine-induced protection, we immunized IL-23p19$^{-/-}$ mice (which fail to generate Th17 cells), IL-12p35$^{-/-}$ mice (no Th1 cells), and IL-12/23p40$^{-/-}$ mice (neither Th1 nor Th17 cells) with our CAF-adjuvanted vaccine before challenge infection. Neither IL-23p19$^{-/-}$ nor IL-12p35$^{-/-}$ mice nor IL-12/23p40$^{-/-}$ mice were able to control the challenge infection as determined by colony counting (Figure 5*C*); all protection correlates (leukocyte, memory T cell, mast cell, neutrophil infiltration) confirmed the defect in developing protective immunity of the 3 strains (Figure 5*D*). Intracellular cytokine staining and quantitative polymerase chain reaction revealed that the gastric infiltration of Th1 cells and gastric IFN-γ production were reduced as expected in the IL-12p35$^{-/-}$ mice and IL-12/23p40$^{-/-}$ mice (and somewhat also in the IL-23p19$^{-/-}$ mice), whereas the gastric numbers of Th17 cells and gastric IL-17 production were reduced in the IL-23p19$^{-/-}$ and IL-12/23p40$^{-/-}$ mice (Figure 5*E, F*). IL-12/23p40$^{-/-}$ mice showed an additive effect with respect to most protection correlates (Figure 5*D–F*). In summary, we conclude that mixed Th1 and Th17 responses are hallmarks of protective immunity conferred by a CAF-adjuvanted *H pylori* vaccine, and that both Th responses are absolutely required for protection.

Regulatory T Cells Impair Protective Immunity to H pylori

None of the currently available vaccine regimens targeting *H pylori* induce sterilizing immunity; this conundrum is one of the main obstacles in *H pylori* vaccine development.[36] Hypothesizing that Tregs might actively counteract anti-*H pylori* immunity by favoring peripheral tolerance to the infection, we depleted FoxP3$^+$ Tregs systemically during the challenge phase of our study protocol in a strain expressing the DT receptor under the control of the *foxp3* promoter.[29] The depletion of Tregs (which was >80% efficient in the stomach as determined at the study end point, Supplementary Figure 3) strongly boosted *H pylori*–specific immunity as determined by lower colony counts (Figure 6*A*), higher gastric infiltration of leukocytes, memory T cells, mast cells, and neutrophils (Figure 6*B*) and higher numbers of gastric IFN-γ$^+$ and IL-17$^+$ T cells (Figure 6*C, D*). A more stringent control of the challenge infection was also observed in naïve mice upon depletion of Tregs (Figure 6*A–D*).

Peripheral tolerance to non–self-antigens in the gastrointestinal tract is induced and maintained by DCs; therefore, we speculated that DCs might have an important role in tolerance induction to *H pylori*. We characterized the tolerogenic/immunoregulatory potential of DCs from infected mice ex vivo by testing their ability to convert naïve CD4$^+$ T cells to FoxP3$^+$ Treg in a transforming growth factor–β-dependent manner. CD11c$^+$ cells were enriched by immunomagnetic sorting from single-cell MLN preparations of infected mice, uninfected controls, or immunized/infected mice. The resulting >80% pure DC preparations were then cocultured with splenic CD4$^+$CD25$^-$ T cells from naïve mice under conditions of CD3 activation and transforming growth factor–β and IL-2 exposure (Figure 6*E*). DCs from infected animals induced FoxP3$^+$ Treg more efficiently than DCs from uninfected controls or from immunized/infected mice (Figure 6*E*). The ex vivo–induced Treg further expressed high levels of IL-10 as assessed by intracellular cytokine staining (Figure 6*F*). In conclusion, the exposure of mice to live *H pylori* generates MLN DCs with tolerogenic properties that are very efficient inducers of Tregs; Tregs in turn actively inhibit *H pylori* clearance and their depletion enhances *H pylori*–specific protective immunity.

Depletion of DCs During H pylori *Challenge Improves Vaccine-Induced Protective Immunity*

As DCs are known to induce tolerance in the gastrointestinal tract and DCs from infected mice are more potent inducers of Tregs than DCs from uninfected

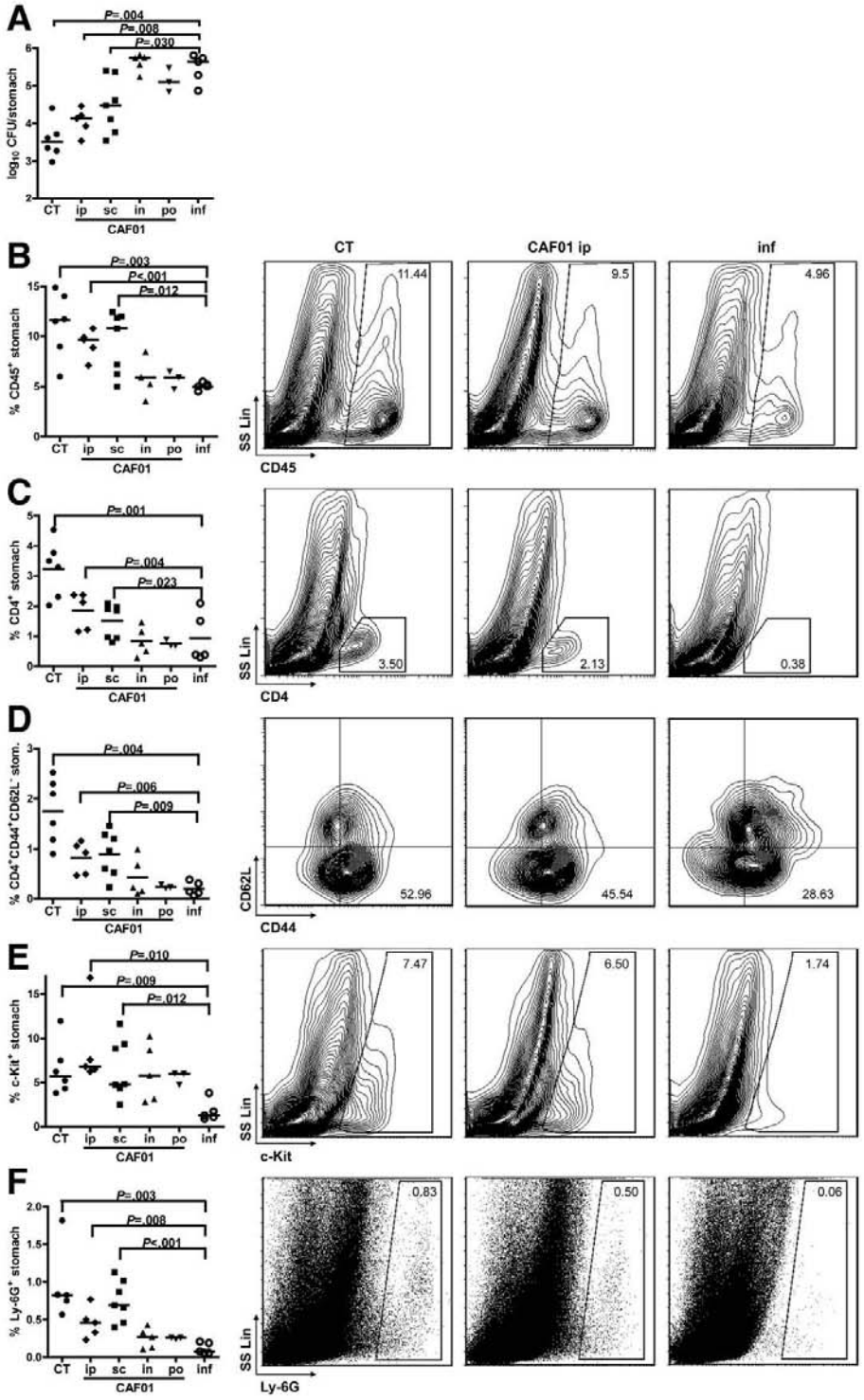


Figure 4. CAF01 vaccine-induced protection is accompanied by gastric infiltration of various immune cell types. (*A*) Colonization levels of the mice analyzed with respect to gastric infiltration in *panels B–F*. (*B–F*) Gastric immune cell infiltration in % of total stomach cells (*left panels*) and representative flow cytometry plots (*right panels*) of CT-immunized, CAF01-immunized, and infected-only (inf) mice. Leukocytes (CD45+, *B*), CD4+ T cells (*C*), activated memory CD4+ T cells (CD44+CD62L−; *D*), mast cells (c-Kit+; *E*), and neutrophils (Ly6-G+; *F*) are shown. In *panel D*, the plots show the CD44+CD62L− fraction of all gastric CD4+ cells. *Panels A–F* show data from 2 combined experiments.

donors, we postulated that the systemic depletion of DCs during the challenge phase of our immunization protocol should improve vaccine-induced protection. To test this possibility, we immunized mice expressing the DT receptor under the control of the DC-specific *cd11c* promoter (*cd11c*-DTR tg) and depleted DCs systemically during the

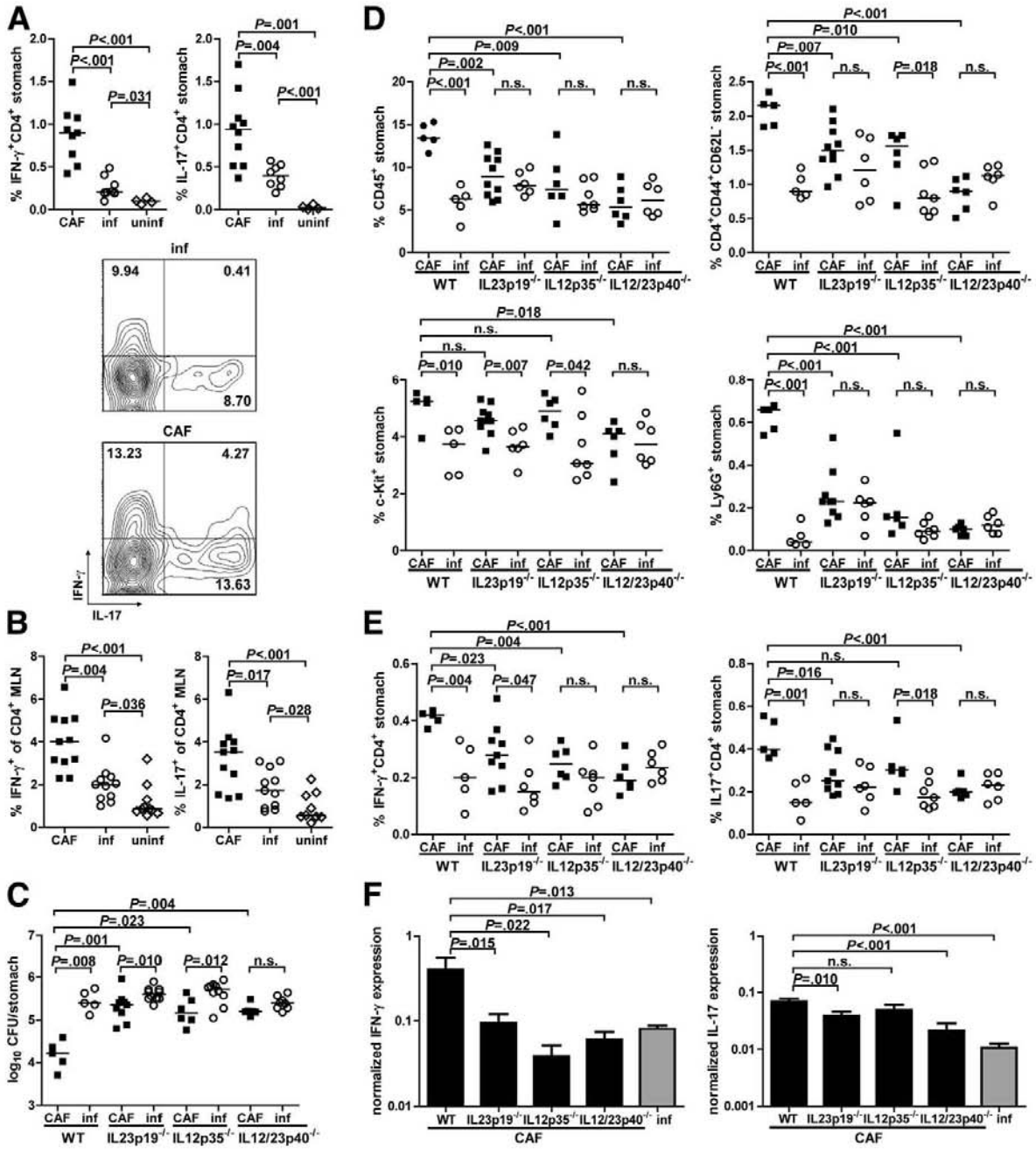


Figure 5. Mixed Th1/Th17 responses against *H pylori* are elicited by a CAF01-adjuvanted vaccine and both Th subsets are required for protection. (*A*) IFN-γ– and IL-17–producing CD4+ T cells in % of all stomach cells in CAF01-immunized, infected, and uninfected wild-type mice as determined by intracellular cytokine staining (pooled data from 2 independent experiments; *upper panels*). Representative flow cytometry plots are also shown (*lower panels*). (*B*) IFN-γ– and IL-17–positive fractions of total CD4+ T cells in MLN of CAF01-immunized, infected, and uninfected mice. (*C*) Colonization levels of wild-type, IL-23p19−/−, IL-12p35−/−, and IL-12/23p40−/− mice immunized SC with CAF01, relative to infected-only controls. (*D, E*) Gastric infiltration in percent of total stomach cells of leukocytes (CD45+), activated memory CD4+ T cells (CD44+CD62L−), mast cells (c-Kit+), and neutrophils (Ly6-G+), (*D*) as well as IFN-γ– and IL-17–producing CD4+ T cells (*E*) of the mice shown in *panel C*. (*F*) Real-time reverse transcription polymerase chain reaction results, normalized to glyceraldehyde-3-phosphate dehydrogenase, for IFN-γ and IL-17 expression in immunized wild-type, IL-23p19−/−, IL-12p35−/−, and IL-12/23p40−/− mice relative to infected-only (inf) wild-type controls.

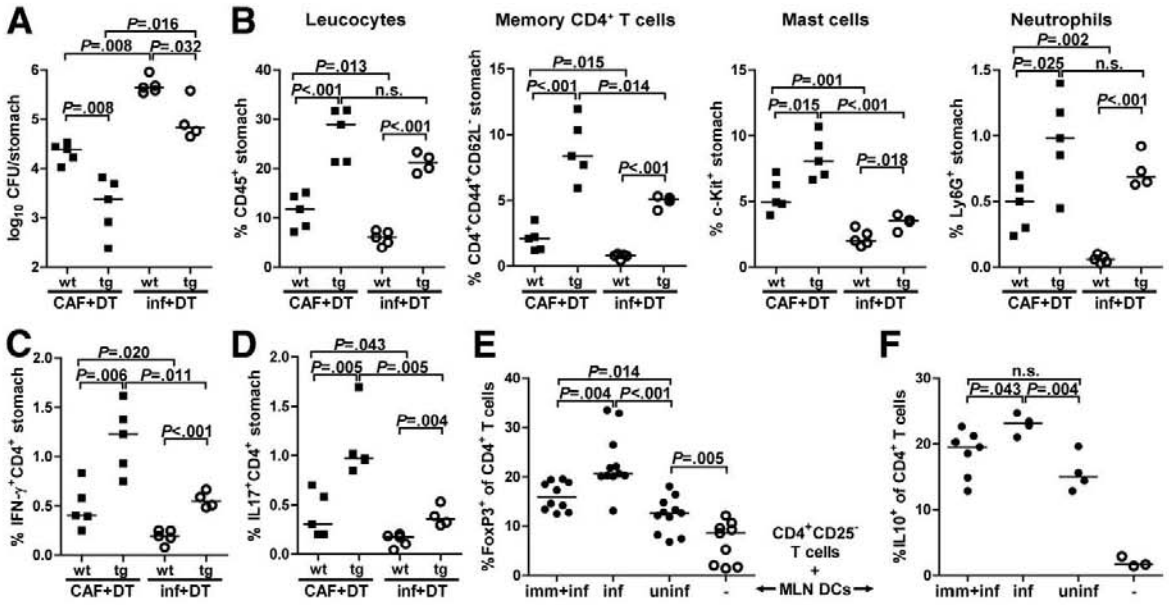


Figure 6. Treg depletion during challenge infection improves vaccine-induced protection; DCs from *H pylori*–infected mice induce Treg ex vivo. (*A*) Colonization levels of *FoxP3*-EGFP-DTR tg and wild-type mice vaccinated SC with CAF01, relative to infected controls; all mice received DT. (*B–D*) Gastric infiltration in percent of total stomach cells of leukocytes ($CD45^+$), activated memory $CD4^+$ T cells ($CD44^+CD62L^-$), mast cells (c-Kit^+), and neutrophils (Ly6-G^+; *B*) as well as IFN-γ– and IL-17–producing $CD4^+$ T cells (*C–D*) for the mice shown in *panel A*. Proportions of $FoxP3^+$ (*E*), and IL-10^+ (*F*) $CD4^+$ T cells converted from naïve $CD4^+CD25^-$ T cells upon coculture with MLN-derived DCs from immunized, infected, or uninfected mice or cultured without DCs. Pooled data from 3 independent experiments are shown in *panel E*.

challenge phase by regular IP administration of DT. Under conditions of DT administration in 2-day intervals, a frequency that was well-tolerated by the animals, the efficiency of DC depletion at all study end points was ~50% in the stomach, MLN, and spleen (Supplementary Figure 4 and data not shown). Interestingly, mice with such reduced DC populations were able to control the infection significantly better than untreated mice or nontransgenic DT-treated littermates, a phenomenon that was observed consistently in both the CT- and CAF01-immunized treatment arms (Figure 7*A*). In accordance with this finding, increased amounts of $CD45^+$ leukocytes, memory T cells, neutrophils, and mast cells were detected in gastric mucosal single-cell preparations of immunized mice with reduced DC populations compared to nondepleted immunized controls (Figure 7*B*). Generally stronger immune infiltration into the gastric mucosa was also observed histologically (Figure 7*C*); DT-depleted immunized mice further exhibited stronger IFN-γ and IL-17 responses in the gastric mucosa and in the MLN than nondepleted immunized mice as shown by real-time reverse transcription polymerase chain reaction or intracellular cytokine staining (Figure 7*D–E* and Supplementary Figure 5). The partial depletion of DCs during infection of nonimmunized animals did not reduce their bacterial burden or affect any of the other assessed infection correlates (Figure 7*A–E*, and data not shown). Speculating that the relative resistance of SS1-infected naïve mice to (partial) DC depletion was due to the comparatively poor immunogenicity of SS1, we repeated the experimental infection with the parental strain of SS1, PMSS1. PMSS1 retains a fully functional Cag pathogenicity island[37] and, as a consequence, initiates stronger immune activation and causes more severe immunopathology than its less virulent, mouse-adapted derivative.[37] DC depletion during PMSS1 infection indeed resulted in lower colonization levels and higher gastric infiltration of $CD4^+$ T cells compared to nondepleted controls (Supplementary Figure 6). Taken together, these findings indicate that tolerogenic mechanisms mediated by DCs and regulatory T cells counteract host immunity to *H pylori* and need to be overcome in order to achieve sterilizing protection.

Discussion

In this study, we provide evidence for the efficacy of a novel mycobacterial cell wall–derived adjuvant CAF01 in *H pylori*–specific vaccination. CAF01 is expected to be safe and has recently been approved for clinical testing of a new mycobacterial subunit vaccine formulation. CAF01-mediated protection in the context of *H pylori* infection was clearly restricted to parenteral routes of vaccine delivery, and was further dependent on MHC class II–restricted T cells of both Th1 and Th17 subsets. Our data thus confirm and extend several previous studies reporting that protective immunity to *Helicobacter* in small rodent models does not require humoral responses,[31–33] but rather is entirely $CD4^+$ T-cell–medi-

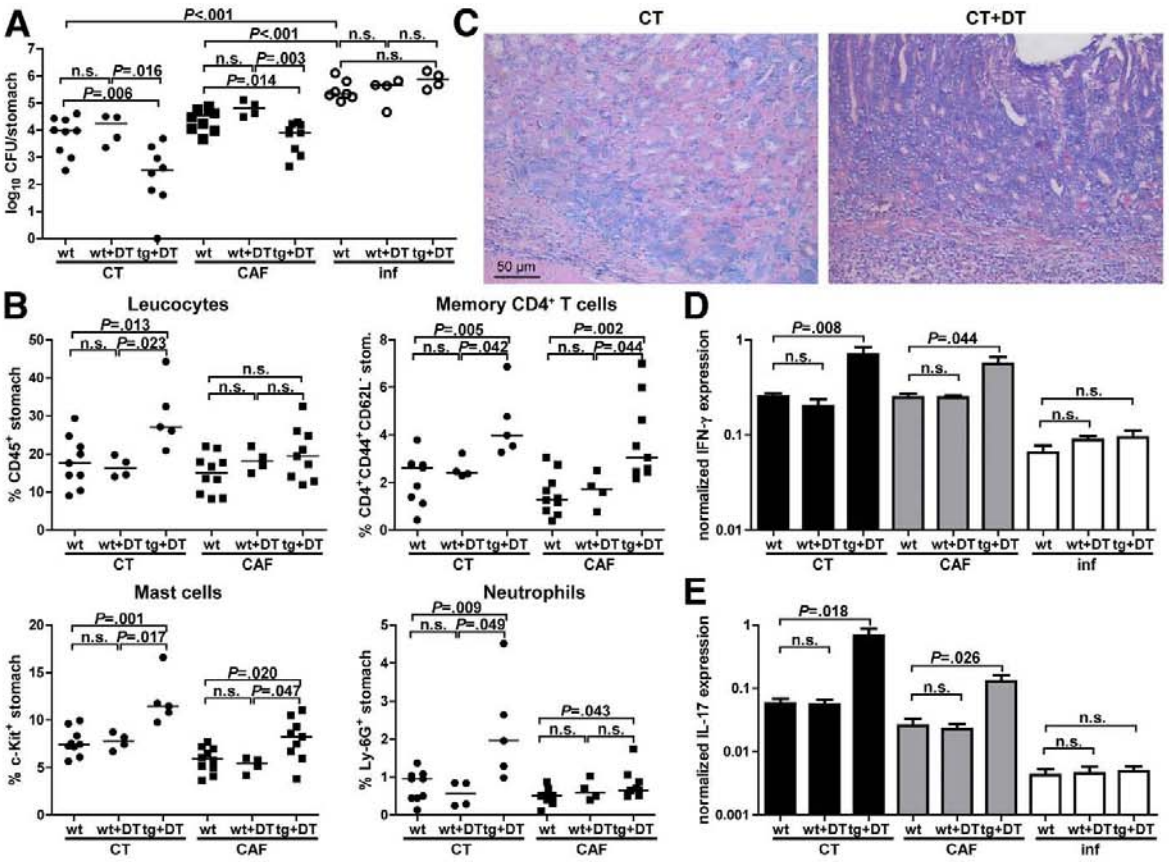


Figure 7. Vaccination outcomes improve upon DC depletion. (*A*) Colonization levels of CT-immunized, CAF01-immunized, and infected-only *CD11c*-DTR tg (tg) compared to wild-type (wt) mice, treated (+DT) or not with DT every 2 days during challenge infection. (*B*) Gastric infiltration of leukocytes ($CD45^+$), memory $CD4^+$ T cells ($CD44^+CD62L^-$), mast cells (c-Kit^+), and neutrophils ($Ly6\text{-}G^+$) in immunized *CD11c*-DTR tg and wild-type mice, treated (+DT) or not with DT. (*C*) Giemsa-stained histological sections of representative CT-immunized and CT-immunized/DC-depleted mice, illustrating immune cell infiltration into the gastric mucosa. (*D–E*) Real-time reverse transcription polymerase chain reaction results, normalized to glyceraldehyde-3-phosphate dehydrogenase, for (*D*) IFN-γ and (*E*) IL-17 expressions in *CD11c*-DTR tg and wild-type immunized and/or infected mice, treated (+DT) or not with DT. Pooled data from 3 independent experiments are shown.

ated[17,38] and dependent on Th1[7,15,39] and/or Th17[5,18] cytokines. By comparing immunological parameters of effective and ineffective delivery routes, we were able to identify a number of correlates that proved to be tightly associated with protection. Gastric mucosal preparations optimized to capture both lamina propria and intraepithelial immune cell infiltrates contained large numbers of mucosa-infiltrating IFN-γ– and IL-17–positive (memory) T cells, mast cells, and neutrophils, suggesting that these populations contribute to the elimination of the bacteria as proposed previously.[5,34]

Possibly the least expected finding of the present study relates to the role of DCs in protective immunity to *H pylori*. We have shown recently that the balance between *H pylori*–specific tolerance and immunity determines disease outcome in a model of gastric preneoplastic pathology induced by a particularly virulent, Cag pathogenicity island-positive *H pylori* isolate.[37] In this disease model, mice that had been infected during the neonatal tolerance window—in which the newborn immune system is immature and naturally skewed toward regulatory rather than immunogenic immune responses—were entirely protected from the immunopathological consequences of infection that are a hallmark of mice infected as adults. In this scenario, DCs are believed to act as important mediators of tolerance induction and maintenance through their ability to induce antigen-specific regulatory T cells in the periphery. In support of this notion, Kao et al recently reported that *H pylori*–infected DCs can suppress Th17 immunity and skew the immune response toward a regulatory phenotype.[40]

Mice that have developed tolerance to the infection are not only protected from gastric immunopathology but are also colonized with *H pylori* at significantly higher densities.[37] This observation prompted us to attempt to improve vaccine efficacy by overruling DC- and Treg-mediated tolerance mechanisms during the challenge phase of our immunization protocols. Indeed, even the partial depletion of all $CD11c^+$ populations that could be

achieved without the lethal side effects that are a hallmark of continuous DT treatment of *CD11c*-DTR-transgenic mice was sufficient to significantly increase the efficacy of both the CAF01- and the CT-adjuvanted vaccines. The reduced bacterial counts recovered from the DC-depleted and Treg-depleted mice were confirmed by the immunological correlates of protection outlined here. The improved protection of DC/Treg-depleted mice appeared to be due to the more efficient induction of T-cellular recall responses. In conclusion, we introduce here a novel vaccine formulation incorporating a mycobacterial cell wall glycolipid that confers Th1/Th17-mediated protective immunity to experimental *H pylori* infection. We further show that the tolerogenic functions of DCs that are activated by live *H pylori* must be overcome in order to achieve optimal protective immunity to this important human gastrointestinal pathogen.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi:10.1053/j.gastro.2011.04.009.

References

1. Marshall BJ, Windsor HM. The relation of *Helicobacter pylori* to gastric adenocarcinoma and lymphoma: pathophysiology, epidemiology, screening, clinical presentation, treatment, and prevention. Med Clin North Am 2005;89:313–344.
2. Mégraud F. *H pylori* antibiotic resistance: prevalence, importance, and advances in testing. Gut 2004;53:1374–1384.
3. Zullo A, Hassan C, Eramo A, et al. Gastric cancer following *Helicobacter pylori* eradication in duodenal ulcer patients. Digest Liver Dis 2006;38:528–529.
4. Rokkas T, Pistiolas D, Sechopoulos P, et al. The long-term impact of *Helicobacter pylori* eradication on gastric histology: a systematic review and meta-analysis. Helicobacter 2007;12(Suppl 2):32–38.
5. DeLyria ES, Redline RW, Blanchard TG. Vaccination of mice against *H pylori* induces a strong Th-17 response and immunity that is neutrophil dependent. Gastroenterology 2009;136:247–256.
6. Mueller A, O'Rourke J, Chu P, et al. Protective immunity against *Helicobacter* is characterized by a unique transcriptional signature. Proc Natl Acad Sci 2003;100:12289–12294.
7. Sayi A, Kohler E, Hitzler I, et al. The $CD4^+$ T cell-mediated IFN-gamma response to *Helicobacter* infection is essential for clearance and determines gastric cancer risk. J Immunol 2009;182:7085–7101.
8. Chen M, Lee A, Hazell S. Immunisation against gastric *Helicobacter* infection in a mouse/*Helicobacter felis* model. Lancet 1992;339:1120–1121.
9. Banerjee S, Medina-Fatimi A, Nichols R, et al. Safety and efficacy of low dose *Escherichia coli* enterotoxin adjuvant for urease based oral immunisation against *Helicobacter pylori* in healthy volunteers. Gut 2002;51:634–640.
10. Sougioultzis S, Lee CK, Alsahli M, et al. Safety and efficacy of *E. coli* enterotoxin adjuvant for urease-based rectal immunization against *Helicobacter pylori*. Vaccine 2002;21:194–201.
11. Kotloff KL, Sztein MB, Wasserman SS, et al. Safety and immunogenicity of oral inactivated whole-cell *Helicobacter pylori* vaccine with adjuvant among volunteers with or without subclinical infection. Infect Immun 2001;69:3581–3590.
12. DiPetrillo MD, Tibbetts T, Kleanthous H, et al. Safety and immunogenicity of phoP/phoQ-deleted *Salmonella typhi* expressing *Helicobacter pylori* urease in adult volunteers. Vaccine 1999;18:449–459.
13. Bumann D, Metzger WG, Mansouri E, et al. Safety and immunogenicity of live recombinant *Salmonella enterica* serovar *Typhi* Ty21a expressing urease A and B from *Helicobacter pylori* in human volunteers. Vaccine 2001;20:845–852.
14. Aebischer T, Bumann D, Epple HJ, et al. Correlation of T cell response and bacterial clearance in human volunteers challenged with *Helicobacter pylori* revealed by randomised controlled vaccination with Ty21a-based Salmonella vaccines. Gut 2008;57:1065–1072.
15. Akhiani AA, Pappo J, Kabok Z, et al. Protection against *Helicobacter pylori* infection following immunization is IL-12-dependent and mediated by Th1 cells. J Immunol 2002;169:6977–6984.
16. Akhiani AA, Schon K, Lycke N. Vaccine-induced immunity against *Helicobacter pylori* infection is impaired in IL-18-deficient mice. J Immunol 2004;173:3348–3356.
17. Ermak TH, Giannasca PJ, Nichols R, et al. Immunization of mice with urease vaccine affords protection against *Helicobacter pylori* infection in the absence of antibodies and is mediated by MHC class II-restricted responses. J Exp Med 1998;188:2277–2288.
18. Velin D, Favre L, Bernasconi E, et al. Interleukin-17 is a critical mediator of vaccine-induced reduction of *Helicobacter* infection in the mouse model. Gastroenterology 2009;136:2237–2246.e1.
19. Rossi G, Ruggiero P, Peppoloni S, et al. Therapeutic vaccination against *Helicobacter pylori* in the beagle dog experimental model: safety, immunogenicity, and efficacy. Infect Immun 2004;72:3252–3259.
20. Gottwein JM, Blanchard TG, Targoni OS, et al. Protective anti-*Helicobacter* immunity is induced with aluminum hydroxide or complete Freund's adjuvant by systemic immunization. J Infect Dis 2001;184:308–314.
21. Eisenberg JC, Czinn SJ, Garhart CA, et al. Protective efficacy of anti–*Helicobacter pylori* immunity following systemic immunization of neonatal mice. Infect Immun 2003;71:1820–1827.
22. Malfertheiner P, Schultze V, Rosenkranz B, et al. Safety and immunogenicity of an intramuscular *Helicobacter pylori* vaccine in noninfected volunteers: a phase I study. Gastroenterology 2008;135:787–795.
23. Werninghaus K, Babiak A, Gross O, et al. Adjuvanticity of a synthetic cord factor analogue for subunit *Mycobacterium tuberculosis* vaccination requires FcRgamma-Syk-Card9-dependent innate immune activation. J Exp Med 2009;206:89–97.
24. Korsholm KS, Agger EM, Foged C, et al. The adjuvant mechanism of cationic dimethyldioctadecylammonium liposomes. Immunology 2007;121:216–226.
25. Kamath AT, Rochat A-F, Christensen D, et al. A liposome-based mycobacterial vaccine induces potent adult and neonatal multifunctional T cells through the exquisite targeting of dendritic cells. PLoS ONE 2009;4:e5771.
26. Davidsen J, Rosenkrands I, Christensen D, et al. Characterization of cationic liposomes based on dimethyldioctadecylammonium and synthetic cord factor from *M. tuberculosis* (trehalose 6,6′-dibehenate)—a novel adjuvant inducing both strong CMI and antibody responses. Biochim Biophys Acta Biomembr 2005;1718:22–31.
27. Agger EM, Rosenkrands I, Hansen J, et al. Cationic liposomes formulated with synthetic mycobacterial cordfactor (CAF01): a versatile adjuvant for vaccines with different immunological requirements. PLoS ONE 2008;3:e3116.
28. Gu H, Zou YR, Rajewsky K. Independent control of immunoglobulin switch recombination at individual switch regions evidenced through Cre-loxP-mediated gene targeting. Cell 1993;73:1155–1164.
29. Lahl K, Loddenkemper C, Drouin C, et al. Selective depletion of Foxp3+ regulatory T cells induces a scurfy-like disease. J Exp Med 2007;204:57–63.

30. Lee A, O'Rourke J, Ungria MCD, et al. A standardized mouse model of *Helicobacter pylori* infection: introducing the Sydney strain. Gastroenterology 1997;112:1386–1397.
31. Blanchard TG, Czinn SJ, Redline RW, et al. Antibody-independent protective mucosal immunity to gastric *Helicobacter* infection in mice. Cellular Immunology 1999;191:74–80.
32. Garhart CA, Nedrud JG, Heinzel FP, et al. Vaccine-induced protection against *Helicobacter pylori* in mice lacking both antibodies and interleukin-4. Infect Immun 2003;71:3628–3633.
33. Sutton P, Wilson J, Kosaka T, et al. Therapeutic immunization against *Helicobacter pylori* infection in the absence of antibodies. Immunol Cell Biol 2000;78:28–30.
34. Velin D, Bachmann D, Bouzourene H, et al. Mast cells are critical mediators of vaccine-induced *Helicobacter* clearance in the mouse model. Gastroenterology 2005;129:142–155.
35. Toller IM, Hitzler I, Sayi A, et al. Prostaglandin E2 prevents *Helicobacter*-induced gastric preneoplasia and facilitates persistent infection in a mouse model. Gastroenterology 2010;138:1455–1467, 1467.e1-4.
36. Mueller A, Sayi A, Hitzler I. Protective and pathogenic functions of T cells are inseparable during the *Helicobacter*-host interaction. Discov Med 2009;8:68–73.
37. Arnold I, Lee JY, Amieva MR, et al. Tolerance rather than immunity protects from *Helicobacter pylori*–induced gastric preneoplasia. Gastroenterology 2011;140:199–209.
38. Pappo J, Torrey D, Castriotta L, et al. *Helicobacter pylori* infection in immunized mice lacking major histocompatibility complex class I and class II functions. Infect Immun. 1999;67:337–341.
39. Taylor JM, Ziman ME, Canfield DR, et al. Effects of a Th1- versus a Th2-biased immune response in protection against *Helicobacter pylori* challenge in mice. Microb Pathog 2007;44:20–27.
40. Kao JY, Zhang M, Miller MJ, et al. *Helicobacter pylori* immune escape is mediated by dendritic cell-induced Treg skewing and Th17 suppression in mice. Gastroenterology 2010;138:1046–1054.

Received August 18, 2010. Accepted April 8, 2011.

Reprint requests

Address requests for reprints to: Anne Müller, PhD, Institute of Molecular Cancer Research, University of Zürich, Winterthurerstr. 190, 8057 Zürich, Switzerland. e-mail: mueller@imcr.unizh.ch; fax: +41 44 635 3484.

Acknowledgments

The authors would like to thank Tim Sparwasser and Regeneron Pharmaceuticals for providing *foxP3*-EGFP-DTR transgenic and interleukin-23p19$^{-/-}$ mice, respectively. We are grateful to Maries van den Broek, Roberto Speck, and Josef Jiricny for critical discussions. Isabelle Arnold, Isabella Toller, Esther Kohler, Vanessa Craig, and Ayca Sayi are thanked for experimental assistance and discussions.

Conflicts of Interest

The authors disclose no conflicts.

Funding

This study was funded by grants from the Cancer League of the Canton of Zurich and the Swiss National Science Foundation (to AM). Additional funding was supplied by the University Research Priority Program in Systems Biology.

Supplementary Methods

Enzyme-Linked Immunosorbent Assay and Real-Time Reverse Transcription Polymerase Chain Reaction

For determination of *H pylori*–specific antibody titers, 96-well enzyme-linked immunosorbent assay plates were coated over night with 10 μg/well of *H pylori* sonicate in carbonate buffer and incubated with 1:10.000 diluted sera or 5 μg mucosal protein extracts; bound antibodies were detected with horseradish peroxidase–coupled goat anti-mouse IgG, IgG1, or IgG2c (all AbD Serotec, Kidlington, UK). IFN-γ secreted by cultured MLN and stomach cells was quantified by enzyme-linked immunosorbent assay (R&D Systems). For real-time RT-PCR, total RNA was isolated from one-sixth of every stomach (antrum and corpus) using NucleoSpin RNA II kits (Macherey-Nagel, Düren, Germany). The corresponding complementary DNA served as a template for real-time polymerase chain reaction performed using the LightCycler 480 SYBR Green I master kit (Roche, Basel, Switzerland). Absolute values of IFN-γ and IL-17 expression were normalized to GAPDH expression (conditions: Tm 55°C, 50 cycles; primers: glyceraldehyde-3-phosphate dehydrogenase fw GAC ATT GTT GCC ATC AAC GAC C / glyceraldehyde-3-phosphate dehydrogenase rv CCC GTT GAT GAC CAG CTT CC, IFN-γ fw CAT GGC TGT TTC TGG CTG TTA CTG / IFN-γ rv GTT GCT GAT GGC CTG ATT GTC TTT, IL-17 fw GCT CCA GAA GGC CCT CAG A / IL-17 rv AGC TTT CCC TCC GCA TTG A).

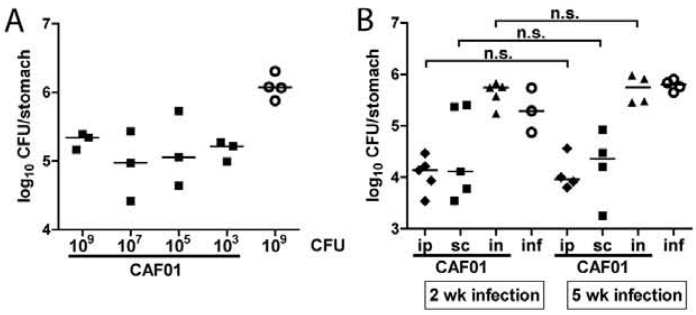
Quantification of H pylori *Colonization by Colony Count Assay*

For the quantitative assessment of *H pylori* colonization, one section of each stomach was transferred to a tube containing Brucella broth and homogenized with an Ultra Turrax homogenizer (John Morris Scientific Ltd, Chatswood, Australia). Serial dilutions were plated on horse blood plates to determine bacterial loads.

Treg Conversion Assay

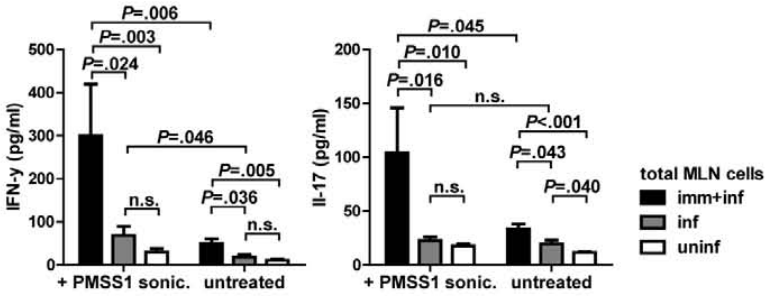
DCs were isolated from MLN single cell suspensions using mouse-specific CD11c microbeads (Miltenyi Biotec). $CD4^+CD25^-$ T cells were prepared from single-cell suspensions of uninfected C57BL/6 spleens by immunomagnetic sorting (R&D Systems). DCs and T cells were cocultured at a ratio of 1:2 (50,000 DCs to 100,000 T cells) in RPMI containing 10% fetal calf serum, 10 ng/mL recombinant transforming growth factor-β (PeproTech, Rocky Hill, NJ), 10 ng/mL recombinant IL-2 (R&D Systems), and 1 μg/mL anti-CD3ε (BD Bioscience). After 72 hours of coculture, the cells were stained first for CD4 and then, after fixation, for FoxP3. The percentage of $FoxP3^+$ $CD4^+$ T cells was assessed by flow cytometric analysis.

Supplemental Figure 1



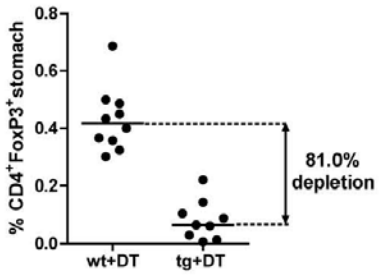
Supplemental Figure 1. The outcome of *H. pylori* challenge infection does not depend on the challenge dose and remains the same at later study endpoints. (*A*), Colonization levels of CAF01 s.c.-immunized mice two weeks after challenge with *H. pylori* doses ranging from 10^3 to 10^9 bacteria and unimmunized controls infected with 10^9 bacteria. (*B*), Colonization levels of mice immunized with CAF01 i.p., s.c. or i.n. as well as unimmunized controls as determined two or five weeks after challenge infection, respectively.

Supplemental Figure 2



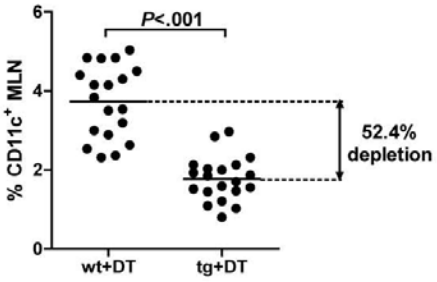
Supplemental Figure 2. ***Helicobacter*-specific response elicited *ex vivo* in MLN cells of mice immunized against and infected with *H. pylori* strain PMSS1.** Total MLN cells were isolated from mice immunized with CAF01 and PMSS1 sonicate and subsequently challenged with PMSS1, as well as infected and uninfected controls. IFN-γ and IL-17 secretion of unstimulated cells and cells restimulated with 10μg/ml PMSS1 sonicate was determined by ELISA after 3 days.

Supplemental Figure 3



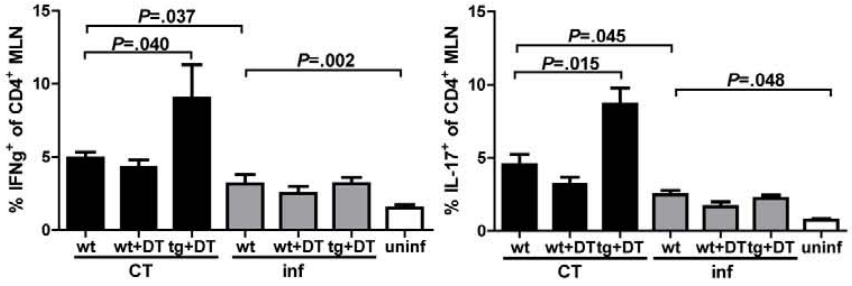
Supplemental Figure 3. Efficiency of Treg depletion. Percentage of $CD4^{+}FoxP3^{+}$ cells in the stomach cell preparations of DT-treated wild type (wt) and *FoxP3*-EGFP-DTR tg (tg) mice.

Supplemental Figure 4



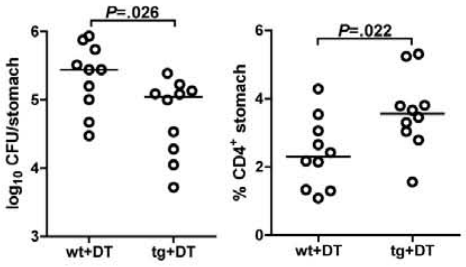
Supplemental Figure 4. Efficiency of DC depletion. Percentage of CD11c$^+$ cells in the MLN of DT-treated wild type (wt+DT) and *CD11c*-DTR tg (tg+DT) mice.

Supplemental Figure 5



Supplemental Figure 5. DC depletion during infection increases IFN-γ- and IL-17-producing CD4$^+$ T cell populations in the MLN of vaccinated mice. Proportion of IFN-γ$^+$ and IL-17$^+$ of total CD4$^+$ T cells in MLN of CT-immunized/infected or only infected wild type (wt) and *CD11c*-DTR tg (tg) mice, respectively, DT-treated (+DT) or not, as well as uninfected controls.

Supplemental Figure 6



Supplemental Figure 6. Depletion of dendritic cells reduces the bacterial burden of PMSS1-infected mice. Colonization levels and gastric $CD4^+$ T cell infiltration of wild type (wt+DT) and *CD11c*-DTR tg (tg+DT) mice infected with PMSS1 and DT-treated during two weeks of infection.

3.3 *H. PYLORI* EXPLOITS AND MANIPULATES INNATE AND ADAPTIVE IMMUNE CELL SIGNALING PATHWAYS TO ESTABLISH PERSISTENCE INFECTION

Published in Cell Communication and Signaling, 2011

Authors: Anne Müller, Mathias Oertli, and Isabelle Arnold

Contributions: I and IA contributed the figures. AM wrote the manuscript

Müller et al. Cell Communication and Signaling 2011, 9:25
http://www.biosignaling.com/content/9/1/25

CELL COMMUNICATION & SIGNALING

REVIEW Open Access

H. pylori exploits and manipulates innate and adaptive immune cell signaling pathways to establish persistent infection

Anne Müller[1*], Mathias Oertli[1] and Isabelle C. Arnold[1,2]

Abstract

Persistent infection with the gastric bacterial pathogen *Helicobacter pylori* causes gastritis and predisposes carriers to a high gastric cancer risk, but has also been linked to protection from allergic, chronic inflammatory and autoimmune diseases. In the course of tens of thousands of years of co-existence with its human host, *H. pylori* has evolved elaborate adaptations that allow it to persist in the hostile environment of the stomach in the face of a vigorous innate and adaptive immune response. For this review, we have identified several key immune cell types and signaling pathways that appear to be preferentially targeted by the bacteria to establish and maintain persistent infection. We explore the mechanisms that allow the bacteria to avoid detection by innate immune cells via their pattern recognition receptors, to escape T-cell mediated adaptive immunity, and to reprogram the immune system towards tolerance rather than immunity. The implications of the immunomodulatory properties of the bacteria for the prevention of allergic and auto-immune diseases in chronically infected individuals are also discussed.

Keywords: immune evasion, innate immune signaling, immunomodulation, persistent infection

Innate immune receptor recognition of *H. pylori*

Innate immune cells as well as epithelial cells forming a first barrier to infection detect invading pathogens via their conserved microbial structures, the so-called pathogen-associated molecular patterns (PAMPs). Examples of PAMPs include microbial nucleid acids, and cell wall and flagellar components such as peptidoglycan, lipopolysaccharide (LPS), lipoproteins and flagellins [1]. PAMPs are recognized by at least four distinct classes of innate immune or pattern recognition receptors (PRRs) that are present either on cytoplasmic and endosomal membranes (Toll-like receptors, TLRs, and C-type lectin receptors, CLRs) or in the cytosol (NOD-like receptors, NLRs, RIG-like receptors, RLRs). Generally speaking, the ligation of TLRs, CLRs or RLRs results in the activation of pro-inflammatory transcription factors in the nucleus; in contrast, most NLRs are involved in the assembly of multi-protein complexes termed "inflammasomes", which process pro-IL-1β and pro-IL-18 to generate the mature, bioactive cytokines [2] (summarized in Figure 1).

PRRs are present not only on hematopoietic cells, but also on gastric epithelial cells which form a first line of defense against *H. pylori* infection [3,4]; therefore, many of the seminal studies in the field have focused on examining *H. pylori* recognition by epithelial cells. In particular, TLR4, 5 and 9 have been detected immunohistochemically on gastric epithelial cells in *H. pylori*-infected as well as uninfected patients, which in principle allows these cells to sense and respond to the infection [3,4]. Interestingly, *H. pylori* differs from other gastrointestinal pathogens in that it has evolved to largely avoid recognition by PAMPs. *H. pylori* flagellin is a poor ligand of TLR5 [5] due to mutations in the TLR5 recognition site of the N-terminal D1 domain of flagellin [6]. Indeed, mutating residues 89-96 of the strongly recognized *Salmonella* flagellin to the corresponding *H. pylori* flaA sequence abolishes TLR5 recognition [6]. The bacterium's LPS consists predominantly of the tetra-acylated lipid A variety, which is known to exhibit 1000-fold reduced bioactivity as compared to *E. coli* LPS [7]. *H. pylori* LPS activates the TLR4/MD-2

* Correspondence: mueller@imcr.uzh.ch
[1]Institute of Molecular Cancer Research, University of Zürich, Zürich, Switzerland
Full list of author information is available at the end of the article

Müller *et al. Cell Communication and Signaling* 2011, **9**:25
http://www.biosignaling.com/content/9/1/25

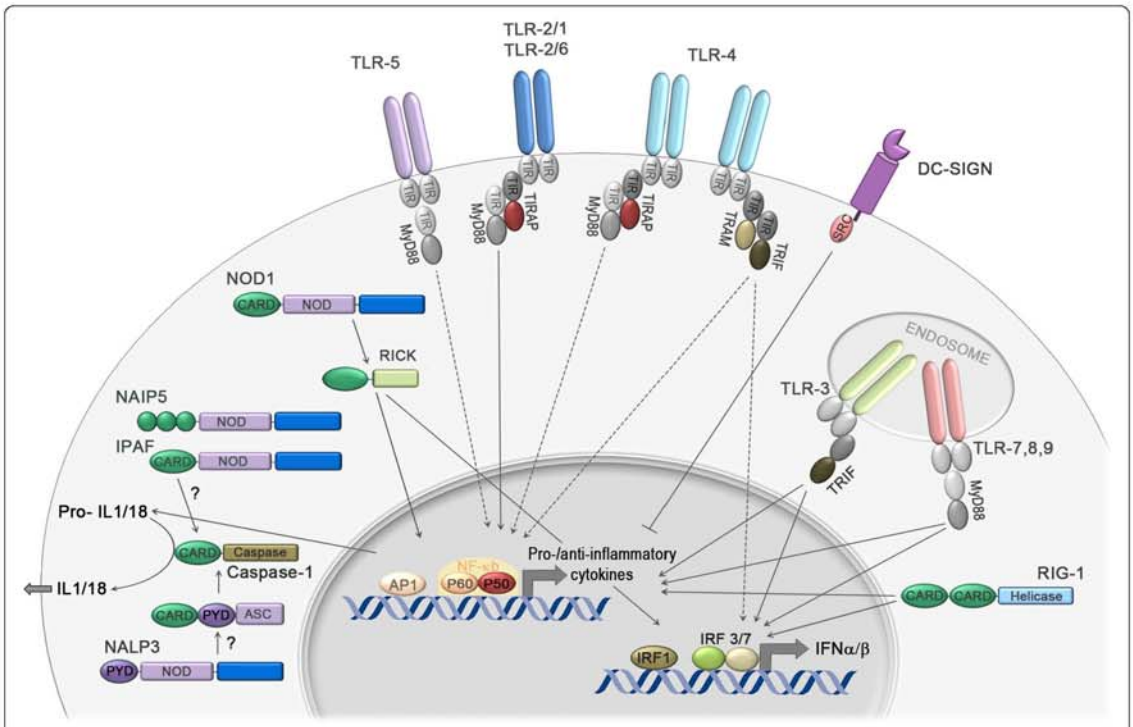


Figure 1 Host innate immune receptors and signaling networks known or believed to participate in *H. pylori* recognition. Toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-like receptors (RLRs), and C-type lectin receptors (CLRs) are expressed on the cell surface, in the endosomes, or in the cytosol of various types of immune cells. Their activation by specific pathogen-associated molecular patterns/ligands induces the expression of cytokine genes or results in the activation of caspase-1 and processing of IL-1β and IL-18. TLR2 and TLR9 recognize *H. pylori* LPS, lipoproteins and DNA, respectively [8-11]; in contrast, *H. pylori* LPS and flagellins are poor ligands of TLR4 and TLR5, respectively (indicated by dotted lines) [5,7]. The RIG-I-dependent recognition of *H. pylori* 5'-triphosphorylated RNA in dendritic cells has been reported to induce expression of type I interferons [10]; fucosylated *H. pylori* ligands for the CLR DC-SIGN have been described, but appear to repress rather than activate signaling downstream of this surface-exposed CLR [12]. NOD1 recognizes *H. pylori* peptidoglycan and activates the transcription factors NF-κB and AP1 [16,17], and also triggers the production of type I interferons [21]. Comparatively little is known regarding additional possible NLR ligands (indicated by question marks). Conceivable, but unconfirmed *H. pylori* NLR ligands include flagellins (for NAIP5 and IPAF) and toxins (NALP3), which would activate the inflammasome and lead to the secretion of mature IL-1β and IL-18. Adapted from [1].

complex, but this has only been shown *in vitro* in cells overexpressing the complex [4]. Rather than being a strong TLR4 ligand, *H. pylori* LPS is thought to activate TLR2 on gastric epithelial cells, but again this has only been demonstrated *in vitro* using ectopic expression of the TLR [8,9]. Animal and cell culture experiments suggest that TLR2 ligands (LPS or other) indeed exist in *H. pylori* and related *Helicobacter* species [9-11], and can bind to TLR2 and activate NF-κB in epithelial cells [9]. However, the net effects of TLR2 ligation are anti-rather than pro-inflammatory *in vivo* as evidenced by lower *H. pylori* colonization levels and more severe immunopathology in gene-targeted mice lacking TLR2 [11]. While *H. pylori* DNA activates TLR9 expressed on dendritic cells (DCs) *in vitro* [10], $TLR9^{-/-}$ mice do not differ from wild type mice in their ability to control *H. pylori* infections [11]. *H. pylori*'s 5'-triphosphorylated RNA can be sensed by the intracellular receptor RIG-1 and triggers type I interferons in DCs [10], but the role of this response in the control of the infection and the infection-associated pathology has to date not been explored. *H. pylori* further possesses fucosylated ligands for the C-type lectin receptor DC-SIGN [12]. However, in contrast to the mannosylated DC-SIGN ligands of *Mycobacterium tuberculosis*, which activate signaling downstream of DC-SIGN and trigger pro-inflammatory cytokine production, the DC-SIGN ligands of *H. pylori* actively dissociate the signaling complex downstream of this C-type lectin to suppress pro-inflammatory cytokine production [12]. The PRRs involved in sensing and responding to *H. pylori* are summarized in Figure 1. Taken together, the data available to date provide ample evidence of the impressive ability of *H. pylori* to avoid

innate immune detection by the host's arsenal of PRRs, thereby preventing innate and adaptive immunity and ensuring its persistence.

NOD1 signaling contributes to anti-*H. pylori* defense mechanisms

As mentioned earlier, the NLRs NOD1 and NOD2 recognize peptidoglycan metabolites of various pathogenic bacteria [13,14] and induce the transcription factor NF-κB to activate immune response genes [15]. Indeed, NOD1 was among the first PRRs shown to recognize an *H. pylori*-derived PAMP, i.e. its peptidoglycan [16], and has since attracted considerable attention. In their initial study, Ferrero and colleagues showed that peptidoglycan recognition by NOD1 was dependent on the expression of a functional Cag pathogenicity island (PAI)-encoded type IV secretion system (T4SS) and resulted in NF-κB activation [16]. More recently, the same group demonstrated that MAP kinases and the AP1 transcription factor were also activated upon peptidoglycan recognition by NOD1 [17]. NOD1 activation by *cag*PAI$^+$ *H. pylori* was further shown to be required for production of beta-defensins by gastric epithelial cells, which contributes to the bactericidal activity of cell culture supernatants against *H. pylori* [18]. *H. pylori* peptidoglycan delivery to cytosolic NOD1 via the T4SS occurs at cholesterol-rich microdomains referred to as lipid rafts, which contain high local concentrations of the T4SS receptor α5β1 integrin [19]; both cholesterol and α5β1 integrin are required for the T4SS-dependent delivery of peptidoglycan [19]. Although it was initially believed that peptidoglycan delivery occurs exclusively via the T4SS [16], Kaparakis *et al.* now provide evidence that outer membrane vesicles prepared from *cag*PAI$^-$ *H. pylori* can also target peptidoglycan to cytosolic NOD1, and that intragastric delivery of peptidoglycan via outer membrane vesicles is sufficient to trigger innate and adaptive immune responses in mice [20].

A novel pathway downstream of NOD1 was recently elucidated in an elegant study by Watanabe *et al.*, who were able to link NOD1 signaling to the production of type I interferons and to the control of *H. pylori* infection [21]. Upon binding to its specific ligand, iE-DAP, NOD1 was shown to induce the sequential activation of the serine threonine kinase RICK, the TNF-associated factor 3 (TRAF3), the kinases TBK1 and IKKε, and ultimately the transcription factor IFN regulatory factor 7 (IRF7) [21]. IRF7 in turn induces IFN-β production, which leads to activation of the heterotrimeric transcription factor complex IFN-stimulated gene factor 3 (ISGF3) and the subsequent production of more IFN-β. Mice lacking the IFN-β receptor exhibit increased susceptibility to *H. pylori* infection, thereby phenocopying the defect of NOD1-deficient mice and suggesting that this pathway participates in host defenses against *H. pylori* [21]. The complete pathway is summarized in Figure 2. The study by Watanabe *et al.* refutes previous work showing that Nod1 activation induces NF-κB activity; at least in epithelial cells, the authors found no or minimal NF-κB activation upon Nod1 ligation by iE-DAP [21]. Another interesting recent study by Liu *et al.* showed that NOD1 and NOD2 are targets of the immunomodulatory glycoprotein olfactomedin 4 (OLFM4) in the context of *H. pylori* infection [22]. OLFM4 is an NF-κB target gene and associates directly with both NOD proteins, thereby creating a negative feedback loop that impairs *H. pylori*-induced NF-κB activation. OLFM4 knockout mice exhibited reduced *H. pylori* loads and enhanced gastric immune cell infiltration compared to wild type animals, suggesting that OLFM4 acts as negative regulator of *H. pylori*-specific, NOD-mediated immune responses [22].

Inhibition of T-cell signaling and proliferation by the *H. pylori* virulence factors VacA and γ-glutamyl-transpeptidase

CD4$^+$ MHC class II-restricted T-cells are absolutely required for the control of experimental *H. pylori* infections and for the development of vaccine-induced protective immunity [23-25]. It therefore does not come as a big surprise that *H. pylori* has evolved virulence factors in the course of its 60.000+ years of co-existence with the human host [26] that allow it to suppress T-cell-mediated immunity. Seminal work by Haas, Cover, Baldari and their co-workers identified the vacuolating cytotoxin (VacA) as a key factor in the *H. pylori*-mediated inhibition of human T-cells [27-29]. VacA had initially been identified due to its ability to induce vacuolization of epithelial cells [30]. Haas, Cover, Baldari and colleagues extended these original findings to show that VacA inhibits cell proliferation by interfering with the T-cell receptor/interleukin-2 (IL-2) signaling pathway at the level of the Ca^{2+}-calmodulin-dependent phosphatase calcineurin [27-29]. VacA thus prevents the nuclear translocation of the T-cell transcription factor NFAT, a global regulator of T-cell responses, resulting in downregulation of IL-2 gene transcription [27,28]. In subsequent studies, Haas and co-workers identified β2 (CD18) integrin to be the relevant receptor for VacA on human T-cells [31]. The authors were able to show that VacA exploits the recycling of lymphocyte function-associated antigen-1 (LFA-1; an integrin heterodimer on T-cells consisting of a β2 subunit associated with the CD11a α subunit) to become internalized by migrating human T-cells. LFA-1-deficient Jurkat T cells were resistant to vacuolation and IL-2 modulation, and genetic complementation restored their sensitivity to VacA [31]. A recent study further showed that VacA uptake depends on protein kinase C-mediated serine/threonine phosphorylation events,

Müller et al. Cell Communication and Signaling 2011, 9:25
http://www.biosignaling.com/content/9/1/25

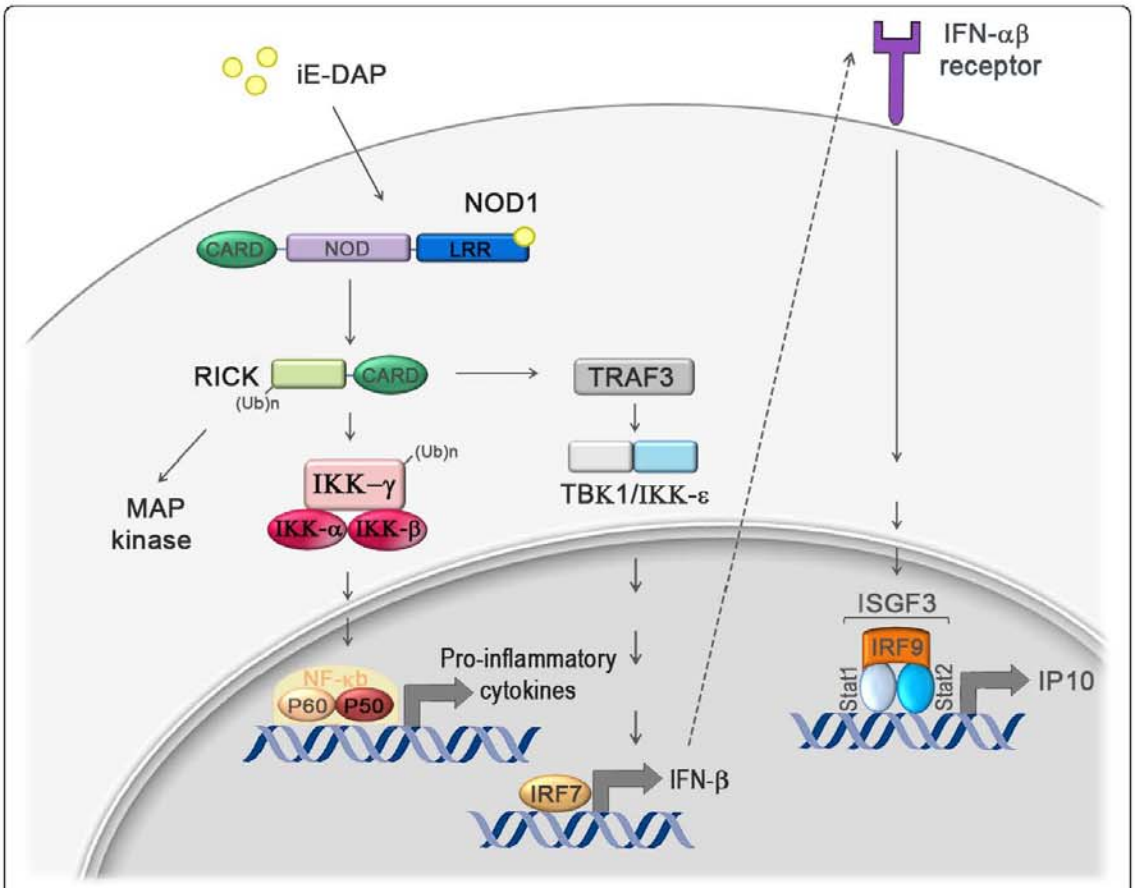


Figure 2 Schematic of NOD1 signaling pathways. NOD1 activation by its specific ligand iE-DAP activates at least two distinct signaling pathways. On the one hand, activated NOD1 triggers IFN-β production through the consecutive activation of RICK, TRAF3, TBK1/IKKε and IRF7. IRF7-dependent IFN-β production leads to the expression of IP-10 and other Th1 cytokines via transactivation of ISGF3 (Stat1-Stat2-IRF9 complex). On the other hand, NOD1 activation triggers the RICK and IKK-α,β,γ complex-dependent activation of NF-κB and subsequent production of proinflammatory cytokines and chemokines such as IL-8. Whether NF-κB is indeed activated upon Nod1 ligation in all cell types, including epithelial cells, remains controversial and will be the subject of future experimentation. Adapted from [56] and [21].

presumably of a specific threonine in the β2/CD18 cytoplasmic tail [32].

An alternative mechanism of T-cell suppression has been proposed by Gerhard and co-workers, who provided evidence in 2005 that human T-cell proliferation is blocked by *H. pylori* without accompanying effects on NFAT activation or cytokine production [33]. The authors postulated at the time that a secreted low-molecular-weight protein distinct from VacA arrests antigen-activated T-cells in the G1 phase of the cell cycle by interfering with G1 cyclin-dependent kinase activity [33]. The authors later demonstrated through a biochemical approach that the secreted γ-glutamyl transpeptidase (GGT) of *H. pylori* is the responsible factor for inhibition of T-cell proliferation; mutagenesis of GGT abrogated the inhibitory effect of the bacteria and recombinantly expressed GGT enzyme showed anti-proliferative activity [34]. The authors concluded from measuring reduced levels of c-Myc and phosphorylated c-Raf protein that GGT induces cell cycle arrest by disrupting the Ras signaling pathway [34].

H. pylori infection induces "tolerogenic" DCs and regulatory T-cells with suppressive activity

Direct inhibition of T-cells by *H. pylori* virulence factors represents a compelling mechanism of immunosuppression and likely contributes to the bacteria's persistence in the human stomach. In addition, several laboratories have reported lately that *H. pylori*-specific effector T-cell responses are under the strict control of regulatory

T-cells (Tregs) in infected humans, and that the depletion of Tregs improves immunological control over the infection and enhances vaccine-induced protective immunity in mouse models [35-38]. The degree to which the infected host generates *H. pylori*-specific Tregs appears to depend largely on the age at the time of infection [35]. Mice that are experimentally infected as adults with virulent *H. pylori* harboring the Cag pathogenicity island-encoded T4SS mentioned earlier rapidly develop gastritis and gastric cancer precursor lesions manifesting histologically as atrophic gastritis, intestinal metaplasia and epithelial hyperplasia [35]. In contrast, mice that are exposed to *H. pylori* during the neonatal period are protected against the development of such lesions, despite the fact that they are colonized at much higher levels than their adult-infected counterparts. The difference in *H. pylori* colonization and the associated pathology is closely linked to dramatic differences in local as well as systemic immune responses to the bacteria between the age groups. Neonatally infected mice develop immunological tolerance to the infection, which prevents them from generating *H. pylori*-specific T-helper responses and protects them from developing T-cell driven immunopathology. *H. pylori*-specific immunological tolerance depends on regulatory T-cells; their systemic depletion breaks tolerance and sensitizes mice to gastric immunopathology [35]. Whether immunological tolerance develops in humans infected early enough with *H. pylori* remains a matter of speculation. A study showing that infected children, but not adults, preferentially generate regulatory T-cell responses to the infection argues in favor of the tolerance model [39].

Above and beyond their role in preventing immunopathological gastric T-helper responses to the infection, Tregs are crucial mediators of the protection against allergen-induced asthma that is conferred by *H. pylori* infection in mice [40] and humans [41-44]. In infected mice subjected to an experimental protocol inducing airway inflammation and hyper-responsiveness through sensitization and challenge with ovalbumin, the protection against asthma can be abrogated by Treg depletion [40]. Conversely, asthma protection can be transferred from infected to uninfected mice by highly purified population of Tregs [40]. The exact mechanism of the suppression of *H. pylori*-specific or of allergen-specific effector T-cells by *H. pylori*-induced regulatory T-cells is not well understood; the secretion of suppressive Treg cytokines such as IL-10 and TGF-β, but also contact-dependent mechanisms of effector T-cell suppression can be envisioned. It is of interest to note in this context that *H. pylori* infection seems to be inversely correlated not only with asthma, but also with chronic inflammatory conditions of the small and large intestine. Several epidemiological studies have pointed out this association, looking at both ulcerative colitis and Crohn's disease patient populations [45,46]. Recent experimental evidence supports this concept; in a model of *Salmonella typhimurium*-induced colitis, concomitant *H. pylori* infection alleviated disease symptoms [47]. Protection against this form of colitis was attributed to reduced Th17 responses in the gut under conditions of co-infection, and coincided with the production of immunomodulatory (Treg-derived?) IL-10 in the mesenteric lymph nodes of the co-infected mice [47].

Inducible Tregs (iTregs; in contrast to "natural", thymus-derived nTregs) differentiate in the periphery as a result of their priming by dendritic cells (DCs) with "tolerogenic" rather than immunogenic properties [48]. Tolerogenic DCs convert naive T-cells into FoxP3$^+$ Tregs through antigen presentation in the absence of co-stimulatory signals or cytokines, either alone or in combination with the production of soluble and membrane-bound tolerogenic factors such as IL-10, TGFβ, retinoic acid, and programmed death ligands [48,49]. Tolerogenic properties have been attributed to immature DCs that have taken up antigen, but have not simultaneously been exposed to TLR or NLR ligands; such DCs are believed to acquire a semi-mature state characterized by high levels of MHCII, but low or no expression of co-stimulatory molecules or pro-inflammatory cytokines [48,49]. It is tempting to speculate based on the characteristic paucity of TLR and NLR ligands that is a hallmark of *H. pylori* that the pathogen has evolved precisely to avoid DC maturation, and to thereby promote Treg rather than T-effector responses. Experimental evidence for such Treg skewing comes from *in vitro* experiments with bone-marrow-derived DCs, which showed that *H. pylori*-experienced DCs appear to preferentially prime Treg over Th17 responses [50] and fail to produce pro-inflammatory cytokines [51]. The model of a predominantly tolerogenic role of DCs in the context of *H. pylori* infection is further corroborated by the demonstration that vaccine-induced protective immunity can be improved by systemic DC depletion [36] and by our finding that the lungs of neonatally infected mice subjected to experimental asthma induction are populated by semi-mature (and presumably tolerogenic) DCs [40].

B-cells exhibit immunoregulatory rather than protective properties in the context of *H. pylori* infection

H. pylori-infected patients are characterized by high serum titers to *H. pylori* antigens, and serology is routinely used to diagnose the infection. Being a mucosal pathogen, *H. pylori* was initially suspected of triggering potentially protective mucosal IgA responses. However, early studies with mice lacking B-cells (μMT$^{-/-}$ mice) or antibodies (IgA$^{-/-}$) revealed that vaccine-induced protective immunity develops equally well in wild type and

Müller *et al. Cell Communication and Signaling* 2011, **9**:25
http://www.biosignaling.com/content/9/1/25

B-cell or antibody-deficient mice [24,52,53]. The results challenged the dogma that extracellular mucosal pathogens are controlled by antibodies and was confirmed again in a recent study introducing a novel and very effective new adjuvant derived from mycobacterial cell walls [36]. Subsequent work by Lycke and colleagues demonstrated that antibodies are not only dispensable for *H. pylori* clearance, but may even have detrimental effects on the human host [52]. $\mu MT^{-/-}$ mice lacking B-cells altogether spontaneously reduce *H. pylori* burdens, but develop accelerated and aggravated gastritis [52]. Gene-targeting of the IL-10 and IgA-encoding genes synergizes to clear the infection, i.e. $IL\text{-}10^{-/-}/IgA^{-/-}$ mice clear *H. pylori* infections better than $IL\text{-}10^{-/-}$ mice, both under conditions of experimental infection of naive as well as previously vaccinated mice [54]. The combined results show rather unequivocally that B-cells impair rather than promote immunity to *H. pylori*.

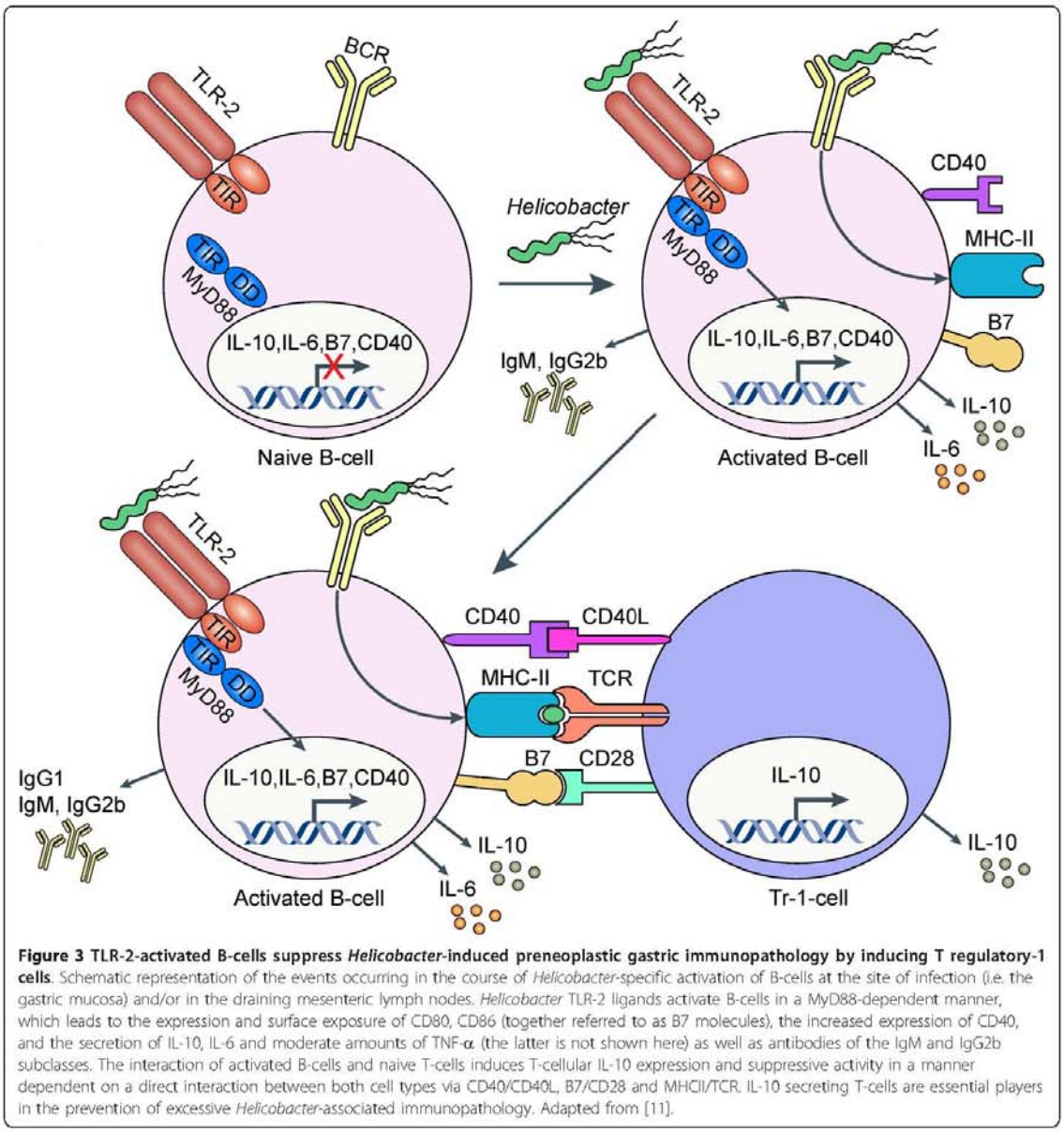


Figure 3 TLR-2-activated B-cells suppress *Helicobacter*-induced preneoplastic gastric immunopathology by inducing T regulatory-1 cells. Schematic representation of the events occurring in the course of *Helicobacter*-specific activation of B-cells at the site of infection (i.e. the gastric mucosa) and/or in the draining mesenteric lymph nodes. *Helicobacter* TLR-2 ligands activate B-cells in a MyD88-dependent manner, which leads to the expression and surface exposure of CD80, CD86 (together referred to as B7 molecules), the increased expression of CD40, and the secretion of IL-10, IL-6 and moderate amounts of TNF-α (the latter is not shown here) as well as antibodies of the IgM and IgG2b subclasses. The interaction of activated B-cells and naive T-cells induces T-cellular IL-10 expression and suppressive activity in a manner dependent on a direct interaction between both cell types via CD40/CD40L, B7/CD28 and MHCII/TCR. IL-10 secreting T-cells are essential players in the prevention of excessive *Helicobacter*-associated immunopathology. Adapted from [11].

We have recently attempted to elucidate the signaling pathways that play a role in B-cellular immunosuppression in the context of *H. pylori* infection. Interestingly, we found that B-cells exposed to *Helicobacter* extract produced large amounts of the regulatory cytokine IL-10 [11]. B-cell recognition of *Helicobacter* and the associated IL-10 production was entirely dependent on TLR2 and Myd88, as B-cells from the respective knock-out mice did not produce IL-10. *Helicobacter*-exposed B-cells further acquired the ability to efficiently induce IL-10 production in co-cultured, naive $CD4^+$ T-cells, thereby converting the T-cells to T-regulatory-1 (Tr1)-like cells with suppressive activity. *In vivo* studies using conditional knock-out mouse strains revealed that IL-10 production by T-cells, but not B-cells, was essential for the suppression of excessive gastric immunopathology. On the other hand, B-cells lacking Myd88 or TLR2 -in contrast to wild type B-cells- were incapable of preventing the characteristic infection-associated immunopathology of $Myd88^{-/-}$ or $IL\text{-}10^{-/-}$ strains when adoptively co-transferred with Tr1 cells [11]. Taken together, the results (summarized in Figure 3) suggest that the B-cell/Tr-1 cell axis is essential for balancing the control of *Helicobacter* infection with the prevention of excessive T-cell-driven gastric immunopathology.

Conclusions

H. pylori has co-evolved with its human host for at least 30.000 years [55]. In contrast to most other bacterial pathogens, which temporarily cause virulent disease but are then rapidly cleared upon the onset of a pathogen-specific adaptive immune response, *H. pylori* persists in its host for decades, if not for life. This extraordinary ability to thrive in the face of a robust and vigorous local and systemic immune response is due to elaborate evolutionary adaptations of *H. pylori* that allow the bacteria to not only escape detection by pattern recognition receptors on innate immune cells, but to also evade adaptive immunity. The immunomodulatory properties of the pathogen reprogram the immune system towards immunological tolerance and assist the bacteria in establishing persistent infection. A byproduct of *H. pylori*-specific immunomodulation and immune tolerance is evident in Western societies that have largely eliminated the infection due to reduced transmission rates, frequent use of antibiotics in childhood and better sanitation. In these populations, the rates of asthma, allergies and other chronic inflammatory and auto-immune diseases have reached epidemic proportions; the inverse correlation of the incidence of such diseases and *H. pylori* infection rates is striking and deserves further investigation. A better understanding of the signaling pathways and molecular players targeted by *H. pylori* to manipulate the host immune response and establish and maintain persistence will be instrumental for improving rational *H. pylori* vaccine design and possibly for exploiting *H. pylori*'s protective properties for asthma and allergy prevention and treatment.

Acknowledgements
Work in the laboratory of A.M. is supported by the University of Zurich Research Priority Program in Systems Biology, the Swiss National Science Foundation and the Swiss and Zurich Cantonal Cancer Leagues.

Author details
[1]Institute of Molecular Cancer Research, University of Zürich, Zürich, Switzerland. [2]Sir William Dunn School of Pathology, University of Oxford, Oxford, UK.

Authors' contributions
AM wrote the manuscript; ICA and MO contributed the figures. All authors have read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Received: 28 July 2011 Accepted: 1 November 2011
Published: 1 November 2011

References

1. Ishii KJ, Koyama S, Nakagawa A, Coban C, Akira S: **Host innate immune receptors and beyond: making sense of microbial infections.** *Cell Host Microbe* 2008, **3**:352-363.
2. Brodsky IE, Monack D: **NLR-mediated control of inflammasome assembly in the host response against bacterial pathogens.** *Semin Immunol* 2009, **21**:199-207.
3. Schmausser B, Andrulis M, Endrich S, Lee SK, Josenhans C, Muller-Hermelink HK, Eck M: **Expression and subcellular distribution of toll-like receptors TLR4, TLR5 and TLR9 on the gastric epithelium in Helicobacter pylori infection.** *Clin Exp Immunol* 2004, **136**:521-526.
4. Ishihara S, Rumi MA, Kadowaki Y, Ortega-Cava CF, Yuki T, Yoshino N, Miyaoka Y, Kazumori H, Ishimura N, Amano Y, Kinoshita Y: **Essential role of MD-2 in TLR4-dependent signaling during Helicobacter pylori-associated gastritis.** *J Immunol* 2004, **173**:1406-1416.
5. Gewirtz AT, Yu Y, Krishna US, Israel DA, Lyons SL, Peek RM Jr: **Helicobacter pylori flagellin evades toll-like receptor 5-mediated innate immunity.** *J Infect Dis* 2004, **189**:1914-1920.
6. Andersen-Nissen E, Smith KD, Strobe KL, Barrett SL, Cookson BT, Logan SM, Aderem A: **Evasion of Toll-like receptor 5 by flagellated bacteria.** *Proc Natl Acad Sci USA* 2005, **102**:9247-9252.
7. Moran AP, Lindner B, Walsh EJ: **Structural characterization of the lipid A component of Helicobacter pylori rough- and smooth-form lipopolysaccharides.** *J Bacteriol* 1997, **179**:6453-6463.
8. Smith MF Jr, Mitchell A, Li G, Ding S, Fitzmaurice AM, Ryan K, Crowe S, Goldberg JB: **Toll-like receptor (TLR) 2 and TLR5, but not TLR4, are required for Helicobacter pylori-induced NF-kappa B activation and chemokine expression by epithelial cells.** *J Biol Chem* 2003, **278**:32552-32560.
9. Chaouche-Drider N, Kaparakis M, Karrar A, Fernandez MI, Carneiro LA, Viala J, Boneca IG, Moran AP, Philpott DJ, Ferrero RL: **A commensal Helicobacter sp. of the rodent intestinal flora activates TLR2 and NOD1 responses in epithelial cells.** *PLoS One* 2009, **4**:e5396.
10. Rad R, Ballhorn W, Voland P, Eisenacher K, Mages J, Rad L, Ferstl R, Lang R, Wagner H, Schmid RM, *et al*: **Extracellular and intracellular pattern recognition receptors cooperate in the recognition of Helicobacter pylori.** *Gastroenterology* 2009, **136**:2247-2257.
11. Sayi A, Kohler E, Toller IM, Flavell RA, Muller W, Roers A, Muller A: **TLR-2-activated B cells suppress Helicobacter-induced preneoplastic gastric immunopathology by inducing T regulatory-1 cells.** *J Immunol* 2011, **186**:878-890.
12. Gringhuis SI, den Dunnen J, Litjens M, van der Vlist M, Geijtenbeek TB: **Carbohydrate-specific signaling through the DC-SIGN signalosome tailors immunity to Mycobacterium tuberculosis, HIV-1 and Helicobacter pylori.** *Nat Immunol* 2009, **10**:1081-1088.
13. Chamaillard M, Hashimoto M, Horie Y, Masumoto J, Qiu S, Saab L, Ogura Y, Kawasaki A, Fukase K, Kusumoto S, *et al*: **An essential role for NOD1 in**

host recognition of bacterial peptidoglycan containing diaminopimelic acid. *Nat Immunol* 2003, **4**:702-707.
14. Girardin SE, Boneca IG, Carneiro LA, Antignac A, Jehanno M, Viala J, Tedin K, Taha MK, Labigne A, Zahringer U, *et al*: **Nod1 detects a unique muropeptide from gram-negative bacterial peptidoglycan.** *Science* 2003, **300**:1584-1587.
15. Kim YG, Park JH, Shaw MH, Franchi L, Inohara N, Nunez G: **The cytosolic sensors Nod1 and Nod2 are critical for bacterial recognition and host defense after exposure to Toll-like receptor ligands.** *Immunity* 2008, **28**:246-257.
16. Viala J, Chaput C, Boneca IG, Cardona A, Girardin SE, Moran AP, Athman R, Memet S, Huerre MR, Coyle AJ, *et al*: **Nod1 responds to peptidoglycan delivered by the Helicobacter pylori cag pathogenicity island.** *Nat Immunol* 2004, **5**:1166-1174.
17. Allison CC, Kufer TA, Kremmer E, Kaparakis M, Ferrero RL: **Helicobacter pylori induces MAPK phosphorylation and AP-1 activation via a NOD1-dependent mechanism.** *J Immunol* 2009, **183**:8099-8109.
18. Grubman A, Kaparakis M, Viala J, Allison C, Badea L, Karrar A, Boneca IG, Le Bourhis L, Reeve S, Smith IA, *et al*: **The innate immune molecule, NOD1, regulates direct killing of Helicobacter pylori by antimicrobial peptides.** *Cell Microbiol* **12**:626-639.
19. Hutton ML, Kaparakis-Liaskos M, Turner L, Cardona A, Kwok T, Ferrero RL: **Helicobacter pylori exploits cholesterol-rich microdomains for induction of NF-kappaB-dependent responses and peptidoglycan delivery in epithelial cells.** *Infect Immun* 2010, **78**:4523-4531.
20. Kaparakis M, Turnbull L, Carneiro L, Firth S, Coleman HA, Parkington HC, Le Bourhis L, Karrar A, Viala J, Mak J, *et al*: **Bacterial membrane vesicles deliver peptidoglycan to NOD1 in epithelial cells.** *Cell Microbiol* **12**:372-385.
21. Watanabe T, Asano N, Fichtner-Feigl S, Gorelick PL, Tsuji Y, Matsumoto Y, Chiba T, Fuss IJ, Kitani A, Strober W: **NOD1 contributes to mouse host defense against Helicobacter pylori via induction of type I IFN and activation of the ISGF3 signaling pathway.** *J Clin Invest* **120**:1645-1662.
22. Liu W, Yan M, Liu Y, Wang R, Li C, Deng C, Singh A, Coleman WG Jr, Rodgers GP: **Olfactomedin 4 down-regulates innate immunity against Helicobacter pylori infection.** *Proc Natl Acad Sci USA* 2010, **107**:11056-11061.
23. Akhiani AA, Pappo J, Kabok Z, Schon K, Gao W, Franzen LE, Lycke N: **Protection against Helicobacter pylori infection following immunization is IL-12-dependent and mediated by Th1 cells.** *J Immunol* 2002, **169**:6977-6984.
24. Ermak TH, Giannasca PJ, Nichols R, Myers GA, Nedrud J, Weltzin R, Lee CK, Kleanthous H, Monath TP: **Immunization of mice with urease vaccine affords protection against Helicobacter pylori infection in the absence of antibodies and is mediated by MHC class II-restricted responses.** *J Exp Med* 1998, **188**:2277-2288.
25. Sayi A, Kohler E, Hitzler I, Arnold I, Schwendener R, Rehrauer H, Muller A: **The CD4+ T cell-mediated IFN-gamma response to Helicobacter infection is essential for clearance and determines gastric cancer risk.** *J Immunol* 2009, **182**:7085-7101.
26. Linz B, Balloux F, Moodley Y, Manica A, Liu H, Roumagnac P, Falush D, Stamer C, Prugnolle F, van der Merwe SW, *et al*: **An African origin for the intimate association between humans and Helicobacter pylori.** *Nature* 2007, **445**:915-918.
27. Gebert B, Fischer W, Weiss E, Hoffmann R, Haas R: **Helicobacter pylori vacuolating cytotoxin inhibits T lymphocyte activation.** *Science* 2003, **301**:1099-1102.
28. Boncristiano M, Paccani SR, Barone S, Ulivieri C, Patrussi L, Ilver D, Amedei A, D'Elios MM, Telford JL, Baldari CT: **The Helicobacter pylori vacuolating toxin inhibits T cell activation by two independent mechanisms.** *J Exp Med* 2003, **198**:1887-1897.
29. Sundrud MS, Torres VJ, Unutmaz D, Cover TL: **Inhibition of primary human T cell proliferation by Helicobacter pylori vacuolating toxin (VacA) is independent of VacA effects on IL-2 secretion.** *Proc Natl Acad Sci USA* 2004, **101**:7727-7732.
30. Cover TL, Blaser MJ: **Purification and characterization of the vacuolating toxin from Helicobacter pylori.** *J Biol Chem* 1992, **267**:10570-10575.
31. Sewald X, Gebert-Vogl B, Prassl S, Barwig I, Weiss E, Fabbri M, Osicka R, Schiemann M, Busch DH, Semmrich M, *et al*: **Integrin subunit CD18 is the T-lymphocyte receptor for the Helicobacter pylori vacuolating cytotoxin.** *Cell Host Microbe* 2008, **3**:20-29.
32. Sewald X, Jimenez-Soto L, Haas R: **PKC-dependent endocytosis of the Helicobacter pylori vacuolating cytotoxin in primary T lymphocytes.** *Cell Microbiol* **13**:482-496.
33. Gerhard M, Schmees C, Voland P, Endres N, Sander M, Reindl W, Rad R, Oelsner M, Decker T, Mempel M, *et al*: **A secreted low-molecular-weight protein from Helicobacter pylori induces cell-cycle arrest of T cells.** *Gastroenterology* 2005, **128**:1327-1339.
34. Schmees C, Prinz C, Treptau T, Rad R, Hengst L, Voland P, Bauer S, Brenner L, Schmid RM, Gerhard M: **Inhibition of T-cell proliferation by Helicobacter pylori gamma-glutamyl transpeptidase.** *Gastroenterology* 2007, **132**:1820-1833.
35. Arnold IC, Lee JY, Amieva MR, Roers A, Flavell RA, Sparwasser T, Muller A: **Tolerance rather than immunity protects from Helicobacter pylori-induced gastric preneoplasia.** *Gastroenterology* 2011, **140**:199-209.
36. Hitzler I, Oertli M, Becher B, Agger EM, Muller A: **Dendritic Cells Prevent Rather Than Promote Immunity Conferred by a Helicobacter Vaccine Using a Mycobacterial Adjuvant.** *Gastroenterology* 2011, **141**:186-196.
37. Lundgren A, Stromberg E, Sjoling A, Lindholm C, Enarsson K, Edebo A, Johnsson E, Suri-Payer E, Larsson P, Rudin A, *et al*: **Mucosal FOXP3-expressing CD4+ CD25high regulatory T cells in Helicobacter pylori-infected patients.** *Infect Immun* 2005, **73**:523-531.
38. Lundgren A, Suri-Payer E, Enarsson K, Svennerholm AM, Lundin BS: **Helicobacter pylori-specific CD4+ CD25high regulatory T cells suppress memory T-cell responses to H. pylori in infected individuals.** *Infect Immun* 2003, **71**:1755-1762.
39. Harris PR, Wright SW, Serrano C, Riera F, Duarte I, Torres J, Pena A, Rollan A, Viviani P, Guiraldes E, *et al*: **Helicobacter pylori gastritis in children is associated with a regulatory T-cell response.** *Gastroenterology* 2008, **134**:491-499.
40. Arnold IC, Dehzad N, Reuter S, Martin H, Becher B, Taube C, Muller A: **Helicobacter pylori infection prevents allergic asthma in mouse models through the induction of regulatory T cells.** *J Clin Invest* 2011, **121**:3088-3093.
41. Blaser MJ, Chen Y, Reibman J: **Does Helicobacter pylori protect against asthma and allergy?** *Gut* 2008, **57**:561-567.
42. Chen Y, Blaser MJ: **Inverse associations of Helicobacter pylori with asthma and allergy.** *Arch Intern Med* 2007, **167**:821-827.
43. Chen Y, Blaser MJ: **Helicobacter pylori colonization is inversely associated with childhood asthma.** *J Infect Dis* 2008, **198**:553-560.
44. Reibman J, Marmor M, Filner J, Fernandez-Beros ME, Rogers L, Perez-Perez GI, Blaser MJ: **Asthma is inversely associated with Helicobacter pylori status in an urban population.** *PLoS One* 2008, **3**:e4060.
45. Pronai L, Schandl L, Orosz Z, Magyar P, Tulassay Z: **Lower prevalence of Helicobacter pylori infection in patients with inflammatory bowel disease but not with chronic obstructive pulmonary disease - antibiotic use in the history does not play a significant role.** *Helicobacter* 2004, **9**:278-283.
46. Sladek M, Jedynak-Wasowicz U, Wedrychowicz A, Kowalska-Duplaga K, Pieczarkowski S, Fyderek K: **[The low prevalence of Helicobacter pylori gastritis in newly diagnosed inflammatory bowel disease children and adolescent].** *Przegl Lek* 2007, **64(Suppl 3)**:65-67.
47. Higgins PD, Johnson LA, Luther J, Zhang M, Sauder KL, Blanco LP, Kao JY: **Prior Helicobacter pylori infection ameliorates Salmonella typhimurium-induced colitis: Mucosal crosstalk between stomach and distal intestine.** *Inflamm Bowel Dis* 17:1398-408.
48. Maldonado RA, von Andrian UH: **How tolerogenic dendritic cells induce regulatory T cells.** *Adv Immunol* 2010, **108**:111-165.
49. Kretschmer K, Apostolou I, Hawiger D, Khazaie K, Nussenzweig MC, von Boehmer H: **Inducing and expanding regulatory T cell populations by foreign antigen.** *Nat Immunol* 2005, **6**:1219-1227.
50. Kao JY, Zhang M, Miller MJ, Mills JC, Wang B, Liu M, Eaton KA, Zou W, Berndt BE, Cole TS, *et al*: **Helicobacter pylori immune escape is mediated by dendritic cell-induced Treg skewing and Th17 suppression in mice.** *Gastroenterology* 2010, **138**:1046-1054.
51. Wang YH, Gorvel JP, Chu YT, Wu JJ, Lei HY: **Helicobacter pylori impairs murine dendritic cell responses to infection.** *PLoS One* 2010, **5**:e10844.
52. Akhiani AA, Schon K, Franzen LE, Pappo J, Lycke N: **Helicobacter pylori-specific antibodies impair the development of gastritis, facilitate bacterial colonization, and counteract resistance against infection.** *J Immunol* 2004, **172**:5024-5033.

53. Garhart CA, Nedrud JG, Heinzel FP, Sigmund NE, Czinn SJ: **Vaccine-induced protection against Helicobacter pylori in mice lacking both antibodies and interleukin-4.** *Infect Immun* 2003, **71**:3628-3633.
54. Akhiani AA, Stensson A, Schon K, Lycke NY: **IgA antibodies impair resistance against Helicobacter pylori infection: studies on immune evasion in IL-10-deficient mice.** *J Immunol* 2005, **174**:8144-8153.
55. Moodley Y, Linz B, Yamaoka Y, Windsor HM, Breurec S, Wu JY, Maady A, Bernhoft S, Thiberge JM, Phuanukoonnon S, *et al*: **The peopling of the Pacific from a bacterial perspective.** *Science* 2009, **323**:527-530.
56. Watanabe T, Asano N, Kitani A, Fuss IJ, Chiba T, Strober W: **NOD1-Mediated Mucosal Host Defense against Helicobacter pylori.** *Int J Inflam* 2010:476482.

doi:10.1186/1478-811X-9-25
Cite this article as: Müller *et al.*: *H. pylori* exploits and manipulates innate and adaptive immune cell signaling pathways to establish persistent infection. *Cell Communication and Signaling* 2011 **9**:25.

4. DISCUSSION

4.1 *HELICOBACTER PYLORI* INFECTIONS INDUCE TOLEROGENIC RATHER THAN IMMUNOGENIC PROPERTIES IN DENDRITIC CELLS

The gram-negative bacterial pathogen *H. pylori* colonizes the gastric mucosa of one half of the world population. The infection is acquired in early childhood and typically persists for life despite the fact that the infected host initiates a vigorous local and systemic immune response to the bacteria [126]. Persistent colonization causes gastritis and predisposes infected individuals to gastric cancer [1, 45, 166, 167]. Conversely, it is also linked to protection from allergic, chronic inflammatory and autoimmune diseases [70, 75, 168]. The majority of infected individuals remain asymptomatic, despite a lifelong persistence of the infection, implicating that *H. pylori* has developed mechanisms to evade immune surveillance and avoid immunopathology.

We hypothesized that *H. pylori* resists immune surveillance by impairing DC function. Indeed, the exposure of BMDCs to live *H. pylori* has a profound impact on the properties of these cells *in vitro*. BMDCs experimentally infected with the pathogen express high levels of major histocompatibility complex (MHC) class II molecules, but surprisingly fail to up-regulate the co-stimulatory molecules cluster of differentiation (CD) 40, CD80 and CD86. Even the induced maturation of BMDCs with *Escherichia coli* (*E. coli*) lipopolysaccharide (LPS) is inhibited in the presence of live *H. pylori*. This inability of the bacteria to induce fully mature BMDCs and to inhibit LPS-provoked maturation is not only observed for the expression of co-stimulatory molecules, but is also evident for the production of pro-inflammatory cytokines such as IL-12p40 and IL-6. In contrast, exposure of BMDCs to *H. pylori* leads to the secretion of the anti-inflammatory cytokine IL-10. A similar pattern can be observed in human PMDCs. Low IL-12 and IL-6 secretion but enhanced expression of IL-10 is detected in *H. pylori*-experienced PMDCs and seems to depend on carbohydrate-specific signaling via the C-type lectin DC-SIGN [95, 96]. Interestingly, we could not attribute the effects of the bacteria on murine BMDCs expressing human DC-SIGN to to this C-type lectin.

One might argue that the low antigenicity of *H. pylori* is the link to its weak effect on BMDC maturation. Various reports highlight the fact that *H. pylori* flagellin is a poor TLR5 ligand, and the bacterium`s LPS consists predominantly of the biologically inactive tetra-

acylated lipid A form [169, 170]. On the other hand, the observation that *H. pylori* inhibits LPS-induced maturation of BMDCs suggests the involvement of a bacterial effector protein rather than only a passive effect due to few, if any, PAMPs. Support for this hypothesis comes from a recent study showing that the purified *H. pylori* effector protein VacA inhibits LPS-induced maturation of BMDCs as assessed by CD40, CD80, CD86 and MHC-II upregulation and the secretion of IL-1β, IL-12p70, and TNF-α. Mechanistically, the authors show that VacA attenuates the LPS-induced down-regulation of the transcription factor E2F1, a critical regulator of DC activation, resulting in inhibition of maturation [171]. Nevertheless there might be a combinatorial effect of the weak immunogenicity of *H. pylori* and bacterial effector proteins responsible for the prevention of BMDC maturation.

Most strikingly, the semi-mature phenotype ($MHCII^{high}CD80^{low}CD86^{low}CD40^{low}$) is not only observed for *H. pylori*-experienced murine BMDCs, but also in biopsies from infected patients. Although the frequency of $DC\text{-}SIGN^{+}CD11c^{+}$ DCs in the gastric mucosa of infected patients is significantly higher than that of their uninfected counterparts, no phenotypical differences in regard to the DC maturation state are observed. The DCs of infected as well as uninfected patients displayed high expression of HLA-DR but no or only little expression of the co-stimulatory molecules CD80, CD83, or CD86. Despite being present in large numbers in the mucosa of infected patients, the recruited DCs display a semi-mature phenotype, adding further support to the inability of *H. pylori* to fully activate DCs.

In theory, DCs have to provide three signals in order to initiate fully-fledged effector T cells. The first signal is the antigenic trigger provided by MHC molecules presenting the cognate peptide, the second signal is provided by co-stimulatory molecules expressed at the surface such as CD40, CD80, CD83 and CD86 and the third signal are cytokines, secreted by either DCs or the microenvironment, that promote and modulate effector T cell functions [92, 137]. Interestingly, DCs providing an antigenic stimulus by MHC molecules to T cells in the absence of concomitant upregulation of co-stimulatory molecules and/or the secretion of certain cytokines have been implicated in immune tolerance by promoting Treg differentiation [137, 154-157, 172]. In *H. pylori*-experienced DCs, we miss two out of three signals, namely high levels of the proinflammatory cytokines IL-12 and IL-6, and efficient up-regulation of the co-stimulatory membrane receptors CD40, CD80 and CD86. Based on these observations, we hypothesized that *H. pylori* renders DCs tolerogenic and that these DCs might be efficient inducers of Tregs.

4.2 *HELICOBACTER PYLORI*-EXPERIENCED DCS INDUCE TREGS RATHER THAN T-EFFECTOR RESPONSES

Several studies report that the maturation status of DCs influences the modulation of effector T cell function. As discussed above, one of the inherent characteristics of DCs providing an antigenic stimulus while lacking co-stimulatory molecules and secretion of pro-inflammatory cytokines is their ability to convert naive T cells into FoxP3-expressing Tregs with a suppressive activity [155-157, 172]. To test the assumption that *H. pylori*-experienced, semi-mature BMDCs not only display a tolerogenic phenotype, but indeed are tolerance-promoting, we tested their ability to induce Tregs *in vitro*. In line with their semi-mature phenotype, *H. pylori*-treated BMDCs induce significantly more FoxP3$^+$ Tregs compared to their untreated counterparts when cultured with naive T cells in the presence of anti-CD3 cross-linking antibody and recombinant TGF-β. In accordance with several studies, the addition of recombinant TGF-β to our co-culture system is necessary to induce and maintain FoxP3 expression [173, 174]. Additionally, direct contact between *H. pylori*-experienced BMDCs and T cells is required for efficient induction of Tregs, as the separation of both cell populations by a trans-well filter abrogated the effect. One might argue that the artificial addition of recombinant TGF-β does not mirror a physiological, *in vivo* situation, but several studies report that the gastric mucosa of *H. pylori*-infected patients is a TGF-β-rich environment if compared to that of healthy individuals [175-178]. In addition to be in a prerequisite for driving Treg induction, TGF-β is known to inhibit DC maturation and down-regulate IL-12 expression, rendering DCs tolerogenic [179, 180]. It is tempting to speculate that this inhibitory effect of TGF-β on DCs might act synergistically with *H. pylori* to render gastric DCs tolerogenic *in vivo*. However it is not clear to what extent the tolerance-promoting capacity of *H. pylori*-experienced DCs relies on TGF- β *in vivo*.

Since BMDCs are a rather artificial cell type which is not found *in vivo*, we immunomagnetically purified CD11c$^+$ DCs from the mesenteric lymph nodes (MLNs) of uninfected C57BL/6 mice to further verify the tolerogenic capacity of *H. pylori*-experienced DCs. MLNs represent one out of two reported sites of *H. pylori*-specific T cell priming alongside the Peyer`s patches [181-184]. We next infected these MLN-derived DCs with live *H. pylori* prior to co-culture with naive T cells. Similar to their more artificial BMDC counterpart, *H. pylori*-treated MLN-derived DCs convert significantly more naive T cells to FoxP3$^+$ Tregs as compared to untreated, control MLN DCs, indicating that the

tolerizing effects of *H. pylori* exposure are common to both BMDC and MLN DC populations. Interestingly, *H. pylori*-infected BMDCs and MLN DCs loaded with ovalbumin are able to trigger the conversion of naive ovalbumin-specific T cells to FoxP3$^+$ Tregs in the absence of anti-CD3 cross-linking antibody, suggesting that the *H. pylori*-induced tolerogenic characteristics of DCs are not *H. pylori*-antigen-specific. The finding that *H. pylori*-experienced DCs are potent inducers of Tregs, are in accordance with a recent study by Robinson *et al.* [123]. The authors report that asymptomatic carriers of *H. pylori* mount a Treg-predominant response to the bacterium, whereas carriers suffering from peptic ulcer disease exhibit strong Th1 and Th2 responses to the infection. In light of these findings we hypothesized that the exposure of DCs to *H. pylori* not only induces Tregs but simultaneously impairs their ability to activate potent T cell effector functions and the induction of Th1 differentiation. Indeed, BMDCs and MLN DCs infected with *H. pylori* are significantly less able than their naive counterparts to induce IFN-γ expression and secretion in OTII T cells activated by either anti-CD3 cross-linking or ovalbumin priming. The combined results suggest that exposure of DCs to the pathogen favours Treg over Th1 differentiation arguing, that strong gastric Treg responses induced by tolerogenic DCs ensure persistent infection in the host while preventing excessive immunopathology *in vivo*.

Robinson *et al.* report that IL-10-expressing Tregs are particularly abundant in the gastric mucosa of asymptomatic carriers compared with patients with peptic ulcer disease. Interestingly, there is a strong association between mucosal IL-10 levels and bacterial densities. High Treg-derived IL-10 expression in the mucosa of asymptomatic carriers directly correlates with high colonization scores. In contrast, carriers suffering from peptic ulcer disease show low levels of mucosal IL-10 and a comparatively low bacterial burden. Additionally, Arnold *et al.* found that mice experimentally infected with *H. pylori* as adults have a relatively low bacterial burden but develop gastritis and gastric cancer precursor lesion, whereas mice infected with *H. pylori* during the neonatal period display a high bacterial colonization and no excessive immunopathology. Thus, *H. pylori* colonization levels and immunopathology are inversely correlated and are linked to drastic differences in local as well as systemic immune responses to the pathogen between the two groups, depending on the age and the time of infection. Whereas immunopathology and the decrease in colonization levels in mice infected as adults depends on Th1-differentiated CD4$^+$IFN-y$^+$ effector T cells, immunological tolerance developed by neonatally infected mice depends on peripherally induced Tregs [122, 185].

The neonatal infection model is therefore particularly interesting in the context of the ability of *H. pylori* to induce tolerogenic DCs *in vivo*. We therefore immunomagnetically isolated MLN DCs from either uninfected mice, mice infected as adults or mice infected during the neonatal period and compared their ability to convert naive T cells to FoxP3-expressing Tregs. MLN DCs from infected mice induce significantly more Tregs than their counterparts from uninfected mice. Interestingly, MLN DCs from neonatally infected, tolerant mice, which score highest in terms of colonization, are particularly good inducers of Tregs but fail to generate Th1 responses against the infection, whereas MLN DCs from adult-infected mice induce less Tregs but show an enhanced Th1 differentiated phenotype. Data gathered in TCR-$\beta^{-/-}$ mice, which lack the α/β T cells altogether, suggest that the pronounced tolerogenic features of MLN DCs from mice infected as neonates is not simply a consequence of higher bacterial colonization levels but rather due to qualitative differences of these DCs compared to their counterparts from adult-infected mice. Neonatal and adult infection in the TCR-$\beta^{-/-}$ background results in similarly high bacterial burden in both age groups due to the complete absence of functional T cells (Fig. 7A). Surprisingly, MLN DCs from neonatally-infected TCR-$\beta^{-/-}$ knock-out mice still induce significantly more Tregs than MLN DCs of their adult-infected counterparts from the same knock-out (Fig. 7B), for reasons that remain unclear at the moment.

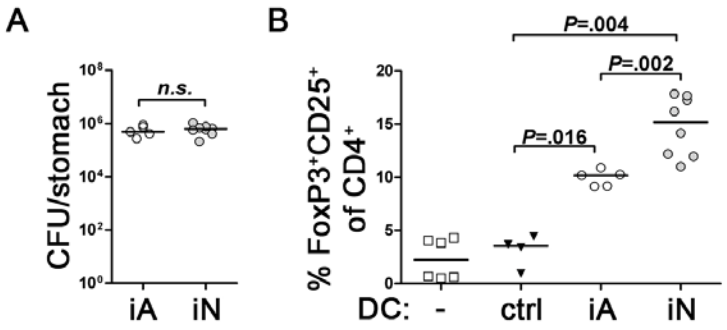


Fig. 7: Adult and neonatal infection of TCR-$\beta^{-/-}$ mice. Colony forming units (CFU) of TCR-$\beta^{-/-}$ mice infected with *H. pylori* at 7 days (iN) or 6 weeks (iA) of age (**A**). Ability of MLN DCs isolated from **A** to convert naive T-cells to FoxP3^{+} Tregs (**B**).

As mentioned above, the results strongly suggest that there is a difference in the quality of DCs isolated from neonatally infected mice compared to MLN DCs from their adult-infected counterparts. In light of these findings comparative gene-expression arrays and

a closer analysis of the different DC sub-populations of the two age groups would be worthwhile. The experiment additionally allows us to answer the "chicken or the egg" question. Tregs and DCs can regulate one another in a negative feedback loop to promote and maintain a local and systemic tolerogenic, immunosuppressive environment. Several mechanisms how Tregs can induce tolerogenic DCs have been proposed. For instance, IL-10 secreted by Tregs (and other sources) can signal via the IL-10 receptor to maintain DCs in their immature state by repressing genes associated with DC maturation and immunogenicity [186, 187]. Additionally, McGuirk *et al.* showed that Tregs can render DCs tolerogenic in an IL-10 dependent manner [165]. As discussed above, Treg-derived TGF-β has similar effects on the maturation status and activation of DCs, although TGF-β signaling induces a much larger repertoire of genes in DCs than IL-10. Besides the induction of TGF-β itself as well as TGF-β receptor and others, the TGF-β-induced transcriptional program in DCs includes IL-18 [188-190]. The crucial requirement for IL-18 for the function of tolerogenic DCs will be discussed later. In addition to the cytokine-induced inhibition of DC maturation, Tregs can form aggregates on DCs, thereby down-regulating their co-stimulatory molecules and maturation markers, thus impairing the ability of DCs to induce antigen-specific T cells [52]. In summary, Tregs and DCs can mutually influence each other, raising the possibility that Tregs, once they are induced in the periphery by *H. pylori*, can render DCs tolerogenic independently of a previous DC-*H. pylori* interaction. Since we can exclude this mechanism in the TCR-$\beta^{-/-}$ background and the MLN DCs isolated from these mice still possess tolerance-promoting features, we can propose that there is a Treg-independent mechanism *in vivo* rendering DCs tolerogenic, probably by a direct modulation of DCs by *H. pylori*. In light of these findings, the TCR-$\beta^{-/-}$ knock-out provides a useful model to test factors (e.g. bacterial factors or pharmacological substances) modulating DC activity in the absence of adaptive immunity. Further support for the hypothesis that *H. pylori*-experienced DCs induce tolerance rather than promote immunity comes from several DC depletion studies in mice. Partial depletion of DCs in *cd11c*DTR-transgenic mice (which express the diphteria toxin receptor under the control of the DC-specific *cd11c* promotor) infected as adults with the highly virulent *H. pylori* strain PMSS1 results in lower colonization levels and enhanced gastric infiltration of $CD4^+$ T cells compared to non-depleted controls (Fig. 8A and B). Additionally, partial DC depletion in a vaccination model of *H. pylori* sonicate-immunized mice improves protective immunity. DC-depleted, immunized mice show a

reduced bacterial burden accompanied by a generally stronger infiltration of immune cells into the gastric mucosa compared to non-depleted, immunized mice. Knowing that

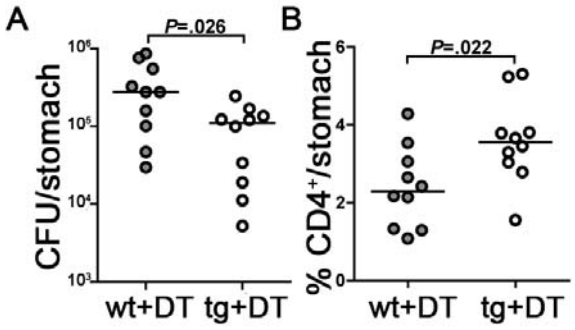


Fig. 8: DC depletion in adult infected mice. Colony forming units (CFU) of C57BL/6 (wt+DT) and *cd11c*-DTR transgenic (tg+DT) mice (**A**). Gastric $CD4^+$ T cell infiltration of mice shown in **A** (**B**). From supplementary figure 6 of section 3.2.

the partial depletion of DCs amplifies T effector over T regulatory responses in adult-infected mice and mice immunized prior to *H. pylori* challenge, we speculated that DCs are required for the development and maintenance of neonatally acquired immunological tolerance as well. Indeed, neonatally infected mice are colonized at significantly lower levels and exhibit higher levels of leukocyte and $CD4^+$ T cell infiltration into their gastric mucosa upon DC-depletion than their DC-replete, infected and uninfected littermates. Although the depletion of $CD11c^+$ DCs breaks neonatal tolerance to some extent, the reduction in the bacterial burden and the paralleled amplification of leukocyte and $CD4^+$ T-cell infiltration is rather modest. We might argue that a complete depletion (rather than 50% incomplete) of DCs over a longer period would lead to a complete protection of the host against the pathogen, as the remaining DCs can still promote tolerance. Additionally, an extended total DC depletion would be necessary to prevent the regeneration of newly induced peripheral Tregs, thereby disrupting the mutual induction of tolerogenic DCs and/or Tregs, respectively. In summary, DCs from infected mice promote Treg over T effector responses and DC-depletion in the *cd11c*-DTR transgenic model reveals that the maintenance of *H. pylori*-specific immune tolerance requires DCs, indicating that *H. pylori*-specific tolerogenic mechanisms are mediated by this cell type *in vivo*. In the face of these findings it becomes clear, that although much effort was put into the development of a sterilizing vaccine against the pathogen, hardly any treatment

regime was ever successful. We therefore postulate that in order to develop a protective vaccine against *H. pylori*, we have to overcome DC- and Treg-mediated tolerance counteracting host immunity to the bacterium.

4.3 HOST REQUIREMENTS FOR *H. PYLORI*-SPECIFIC TOLERANCE

4.3.1 IL-10 AND TGF-β ARE CRUCIAL FOR *H. PYLORI*-SPECIFIC IMMUNE TOLERANCE

Various recent reports show that Tregs and DCs with tolerogenic capacity are necessary for mediating immune tolerance towards *H. pylori*, thereby enabling the pathogen to colonize its host persistently, both in human carriers and in experimentally infected animals [117, 121, 123]. Several host factors have been proposed to be crucial to establish and maintain this state of tolerance. As mentioned above, Robinson *et al.* observed that patients suffering from peptic ulcer disease exhibited strong Th1 and Th2 responses to the pathogen, whereas asymptomatic carriers predominantly mounted a Treg-biased response. The Tregs in the healthy gastric mucosa of asymptomatic patients expressed high levels of IL-10 and received the highest colonization scores. Conversely, peptic ulcer disease patients expressed low levels of Treg-derived IL-10, receiving low scores for bacterial colonization [123]. Additional support for the necessity of IL-10 to promote *H. pylori* persistence on the one hand, and in mediating *H. pylori*-induced immunomodulation on the other hand comes from a study conducted by Harris *et al.*, and from experimental infections of IL-10$^{-/-}$ mice. Harris *et al.* found an inverse correlation between gastric mucosal regulatory cytokine levels and the severity of gastritis. Whereas children which typically exhibit only mild forms of gastritis showed high levels of gastric mucosal Treg numbers together with high levels of the anti-inflammatory cytokines IL-10 and TGF-β, adults with more severe gastritis exhibited low Treg and low regulatory cytokine levels [121]. Furthermore, IL-10$^{-/-}$ mice experimentally infected with *H. pylori* are able to spontaneously clear the infection. Not surprisingly, this clearance is accompanied by strongly enhanced Th1-mediated gastritis [191]. Last but not least, polymorphisms in the IL-10 gene locus have been linked to an increased risk of gastritis and the development of gastric cancer. It is suggested that some polymorphism

of the IL-10 gene locus have a lower IL-10 production and thus increase susceptibility to the disease [192-195]. Several possible sources of IL-10 *in vivo* and/or *in vitro* upon *H. pylori* infections have been proposed. Among them we find gastric mucosal-associated macrophages, DCs and Tregs [87, 95, 96, 118]. In our hands DC-derived IL-10 is not needed to drive differentiation of naive T-cell towards Tregs *in vitro*, since BMDC from IL-10$^{-/-}$ donors are perfectly capable inducing FoxP3 expression. This might not be the case *in vivo* or alternatively, IL-10 is rather crucial for the maintenance of *H. pylori*-specific tolerance once it is established. *H. pylori*-specific tolerance can be maintained by either inhibiting pathogenic T cell responses and/or by rendering bystander DCs tolerogenic (see section 4.2).

Another critical cytokine in the induction of Tregs via *H. pylori*-experienced DCs to establish immune tolerance is TGF-β. Several studies show that high levels of gastric and systemic TGF-β levels correlate with enhanced Treg responses, high colonization burden, and reduced gastric pathology *in vivo* [121, 176]. This correlation is especially evident in the pediatric cohort. Interestingly, Serrano *et al*. provide additional evidence for a participation of TGF-β in the inverse relationship between *H. pylori* infection and the clinical expression of allergy markers in children [121, 176, 196]. Further, we and others show that *H. pylori*-experienced DCs efficiently induce FoxP3 expression in co-cultured naive T cells in a TGF-β-dependent manner [117]. Nevertheless, the main source of TGF-β *in vivo* seems to be gastric epithelial cells and once established Tregs, rather than DCs [175, 197]. Besides having anti-inflammatory capacity by either diminishing Th1 or Th2 responses, TGF-β additionally has a profound impact on Th17 polarisation and on the activation/maturation of DCs [179, 180, 188, 198, 199]. As discussed above, TGF-β can inhibit the maturation of DCs, thereby rendering them tolerogenic. Simultaneously, TGF-β signaling on DCs leads to the expression of IL-18 and is required for the development of Th17 cells [200]. In summary, several lines of evidence indicate that IL-10 and TGF-β are crucial on the host side to maintain *H. pylori*-specific tolerance and to prevent T cell-driven gastric immunopathology.

4.3.2 DC-DERIVED IL-18 DRIVES TREG DIFFERENTIATION, MURINE *H. PYLORI*-SPECIFIC IMMUNE TOLERANCE AND ASTHMA PROTECTION

As discussed above, TGF-β signaling on DCs leads to the expression of IL-18. IL-18 is secreted upon treatment of DCs with *H. pylori*. Interestingly, neither IL-18$^{-/-}$ nor IL-18R$^{-/-}$ mice neonatally infected with *H. pylori* develop full tolerance upon infection. Similar, IL-18$^{-/-}$ mice infected as adults are less densely colonized, but displayed enhanced gastric immunopathology after 1 month of infection with either *H. pylori* or *H. felis* as compared to their wild-type counterparts. In both the neonatal and the adult model of infection, the lack of IL-18 signaling negatively influences Treg numbers and enhances IL-17 expression, but does not influence IFN-γ production in the MLNs. Moreover, in contrast to adoptive transferred CD4^{+}CD25^{+} cells from neonatally infected wild-type mice, the same cell subset from IL-18$^{-/-}$ or IL-18R$^{-/-}$mice does not confer asthma protection in a model of allergen-induced airway hyper-responsiveness. The reduced numbers of Tregs but enhanced Th17 polarization can be linked to DC-derived IL-18 as IL-18$^{-/-}$ DCs lose their tolerogenic features but induce robust Th17 responses in naive T cells upon *H. pylori* infection *in vitro* and *in vivo*. Thus, DC-derived IL-18 seems to be critical in skewing T cell differentiation away from Th17 cells and towards Tregs. Interestingly, the adoptive transfer of the same numbers of either wild type or IL-18$^{-/-}$ CD4^{+}CD25^{+} cells in the asthma model suggests that IL-18 is not only critical to balance the Treg/Th17 ratio, but also for proper suppressive function of Tregs. Since Treg and Th17 cells are developmentally closely linked, the availability of IL-18 might either directly, by binding to its cognate receptor on T cells, or indirectly by its strong potential to induce IFN-γ expression, inhibit Th17 development, thereby shifting the immunological balance from Th17 towards Treg responses [198, 200-202]. Counteracting the IL-18-dependent induction of Treg rather than Th17 responses from a common precursor is IL-1β. IL-1β can either convert Tregs into Th17 or directly induces Th17 cells from IL17R-expressing T cells [200, 203-205]. Surprisingly, both IL-18 and IL-1β are auto-catalytically cleaved by the same cysteine protease, caspase-1, to generate the mature, bio-active cytokines [101, 206].

In summary, IL-18 signaling is critical for DC-induced Treg skewing and Th17 suppression in mice. Additionally, IL-18 is crucial for a proper suppressive function of Tregs.

4.3.3 CASPASE-1 HAS BOTH PROINFLAMMATORY AND REGULATORY PROPERTIES IN *H. PYLORI* INFECTIONS

Caspase-1 is activated upon the NLR-induced assembly of multiprotein complexes called inflammasomes and is critical for the recognition of numerous gram-negative and gram-positive bacteria [207-210]. We showed recently that caspase-1 activation and subsequent release of mature IL-18 and IL-1β is induced in DCs upon *H. pylori* infection *in vitro* and both cytokines show elevated secretion levels in the gastric mucosa of infected mice [211]. Interestingly, caspase-1$^{-/-}$ mice controlled the experimental infection with *H. pylori* more efficiently than their wild type counterparts, being less colonized but displaying increased levels of gastric IL-17, basically phenocopying the disease parameters of experimentally infected IL-18$^{-/-}$ mice. To further elucidate the dependence of the immunoregulatory and/or proinflammatory activities of caspase-1 on IL-18 and IL-1β, we investigated the phenotypes of genetically modified mouse strains lacking either IL-18 or IL-1R infected with *H. pylori*. As discussed above, IL-18$^{-/-}$ mice are less densely colonized, suffer from enhanced gastric immunopathology, and show elevated levels of gastric IL-17 expression as compared to wild-type mice upon infection. In contrast, IL-1R$^{-/-}$ mice experimentally infected with the pathogen display exactly the opposite phenotype. IL-1R$^{-/-}$ are clearly protected against gastritis and gastric preneoplasia that normally arises in wild type mice. The resistance of the IL-1R$^{-/-}$ strain to gastric immunopathology correlated with a high bacterial burden (significantly higher than in wild type mice) and with low gastric expression of IL-17 and IFN-γ [211]. In summary, the results strongly imply that caspase-1 has both proinflammatory and regulatory activities, depending differentially on its two alternative substrates. Whereas the regulatory arm depends on IL-18 mediated suppression of Th17 responses and induction of Tregs, the proinflammatory arm depends on IL-1 signaling, and the differentiation of Th1 and Th17 cell subsets. Which NLRs are involved in the recognition of *H. pylori* leading to caspase-1 activation and IL-1β/IL-18 processing remains to be determined. In this context, it is interesting to note, that *H. pylori* lacks potent TLR ligands whereas the induction of IL-1β expression is MyD88-dependent [205]. This raises the possibility that in the absence of pro-inflammatory TLR-signaling in conjunction with inflammasome activation via NLRs the processing of pre-IL1β and pre-IL-18 is biased towards the latter favouring Treg over Th17 differentiation during *H. pylori* infection. Support for this hypothesis comes from our

recent findings that the addition of *E. coli* LPS can reverse the tolerogenic effect of *H. pylori* on DCs. Since LPS is a strong inducer of IL-1β, which in turn is required for Th17

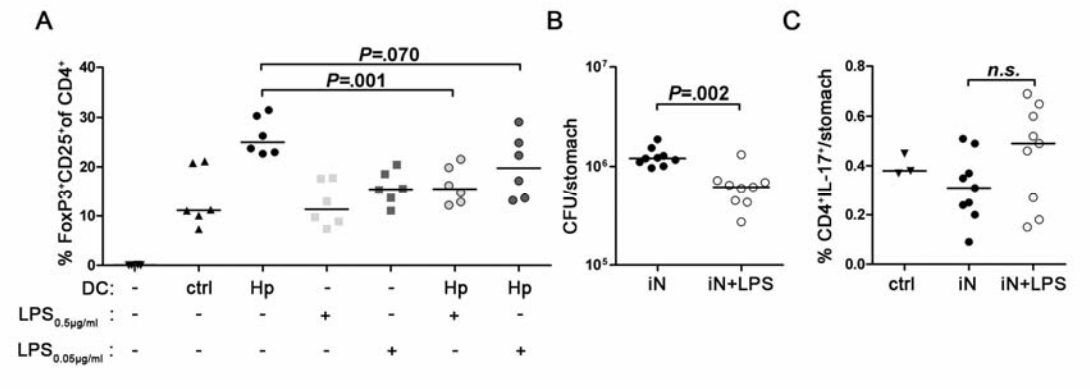


Fig. 9: FoxP3 expression in naive T cells induced by *H. pylori*-experienced DCs can be broken by LPS administration. Capacity of *H. pylori*-experienced BMDC to induce FoxP3 expression in naive T cells in the presence of different LPS concentrations (**A**). Colony forming units (CFU) after administration of 1µg/g bodyweight LPS to neonatal-infected mice (**B**). Infiltrating IL-17$^+$ T cells into the gastric mucosa of mice shown in **B** (**C**).

polarization, the addition of LPS to DCs overrules the tolerizing effects of *H. pylori*, thereby abrogating their capacity to drive FoxP3$^+$ expression in naive T cells (Fig. 9A). Strikingly, intraperitoneal administration of 20 µg/g bodyweight LPS to mice, with pre-established robust neonatal tolerance against the pathogen, is sufficient to reduce bacterial colonization levels in the stomach. Concomitantly, higher percentages of infiltrating IL-17$^+$ T cells into the gastric mucosa of LPS-treated mice are observed as compared to their untreated, infected littermates (Fig. 9B and C), again suggesting that a potent activation of TLR-/Myd88- and NF-κB-dependent expression of IL-1β can influence the relative availability of IL-1β and IL-18, thus dictating whether Tregs or Th17 cells are preferentially induced (Fig 10). *In vivo*, due to the inability of *H. pylori* to efficiently activate TLR-mediated transcription, IL-1β is not available for caspase-1-mediated processing, whereas IL-18 is produced in copious amounts due to efficient inflammasome activation, leading to the preferential induction of Tregs as opposed to Th17 cell subsets. Interestingly, several diallelic polymorphisms have been reported for the *IL-1B* gene encoding for IL-1β. Some of these polymorphisms have been linked to

higher IL-1β production and were associated with an increased risk of gastric cancer and its precursor lesions [53, 212-214]. The gastric cancer

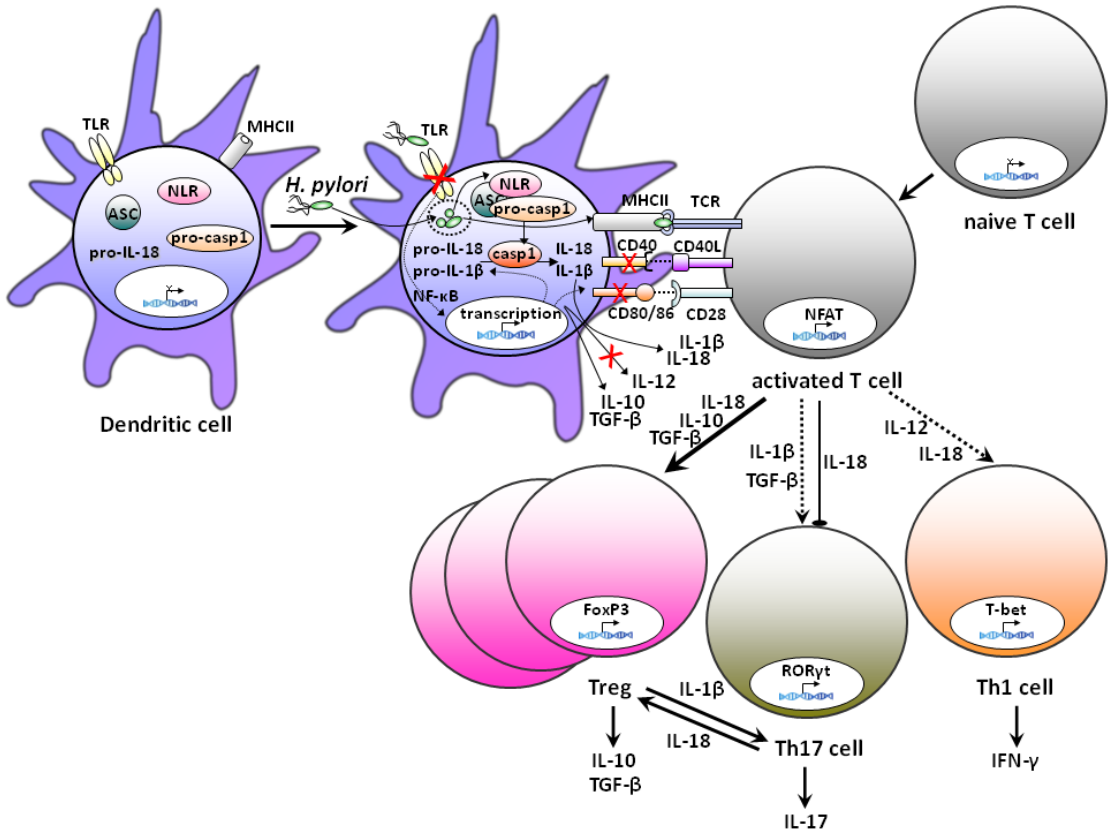


Fig. 10: Overview of the *H. pylori*/DC interaction and the subsequent instruction of naive T cells. Exposure of DCs with *H. pylori* leads to high expression of MHC class II, but only moderate expression of the co-stimulatory molecules CD80, CD86, and CD40, as well as low secretion of the cytokine IL-12. In contrast, copious amounts of IL-10 are made by *H. pylori*-experienced DCs. Recognition of *H. pylori* leads to inflammasome activation through an as yet undetermined NLR(s) resulting in caspase-1 activation and the processing and secretion of large quantities of IL-18 but only modest amounts of IL-1β due to *H. pylori* possessing only weak TLR ligands. *H. pylori*-experienced DCs induce FoxP3$^+$ Tregs in a TGF-β-, IL-18-, and possibly IL-10-dependent manner. In contrast, *H. pylori*-experienced DCs are weak inducers of Th17 and Th1 cell subsets, thereby shifting the balance from Th1/Th17 to Tregs. Figure from [215].

risk is even enhanced in individuals carrying these polymorphisms by *H. pylori* infections, supporting the assumption that excessive production of IL-1β shifts the balance from *H. pylori*-specific tolerance towards Th1-/Th17-driven gastric immunopathology. Based on our data we might assume that infected patients overexpressing IL-1β display lower Treg counts in the gastric mucosa and the draining

lymph nodes. Unfortunately, no data on this correlation are available yet. In summary, *H. pylori*-experienced DCs induce FoxP3$^+$ Tregs in a TGF-β-, IL-18-, and probably IL-10-dependent manner. *H. pylori* recognition leads to inflammasome activation triggering caspase-1-dependent processing of pre-IL-1β and pre-IL18. The relative availability of IL-1β and IL-18 dictates whether Th1/Th17 (high IL-1β, low IL-18) or Treg (low IL-1β, high IL-18) responses are preferentially induced.

4.4 BACTERIAL FACTORS IN DC-MEDIATED TOLERANCE

The observation that *H. pylori* suppresses LPS-induced maturation of BMDCs led us to the hypothesis that the observed semi-mature DC phenotype and their capability to drive FoxP3 expression in naive T cells are not exclusively a consequence of the weak immunogenicity of *H. pylori* but that additional bacterial factor(s) are crucially involved in the instruction of BMDCs to become tolerance-promoting. As discussed above, one promising candidate is VacA due its ability to inhibit LPS induced maturation of BMDCs [(171)]. Additionally, isogenic *vacA* mutants are severely defective in their ability to establish infection and to persistently colonize their host in a mouse model of infection [(216)]. Similarly, the secreted peptidase γ-glutamyl transpeptidase (GGT) has been shown to contribute to the ability of *H. pylori* to colonize the gastric mucosa and establish persistent infection of experimentally infected mice [(217)]. First we compared isogenic Δ*vacA* and Δ*ggt* mutants with the wild type PMSS1 strain with respect to their ability to induce semi-mature BMDCs and their potential to inhibit LPS-induced maturation of BMDCs. Interestingly, BMDCs exposed to the isogenic mutants in different *H. pylori* backgrounds (PMSS1, P12, and G27) expressed similar levels of MHC-II, but significantly higher levels of CD80 than DCs exposed to the corresponding wild type bacteria. Most striking was the observation that the VacA- and GGT-deficient mutants failed to actively suppress the LPS-induced maturation of BMDCs as determined by CD80 expression and secreted IL-12p40 (Fig 11A-B). A possible functional contribution of both factors to *H. pylori* induced DC tolerogenicity was evident in the DC-T cell co-culture system since both mutants exhibited a significant defect in generating DCs with tolerogenic activity (Fig 11C). Furthermore, both the Δ*vacA* and Δ*ggt* mutants showed significant defects in colonizing mice relative to the parental PMSS1 wild type strain after 1 and 2 months of infection as adults (Fig 12A). Interestingly, the lower bacterial burden

of the two mutants was accompanied by stronger Th1 and Th17 responses in the mesenteric lymph nodes of the mutant-infected mice relative to animals infected with the

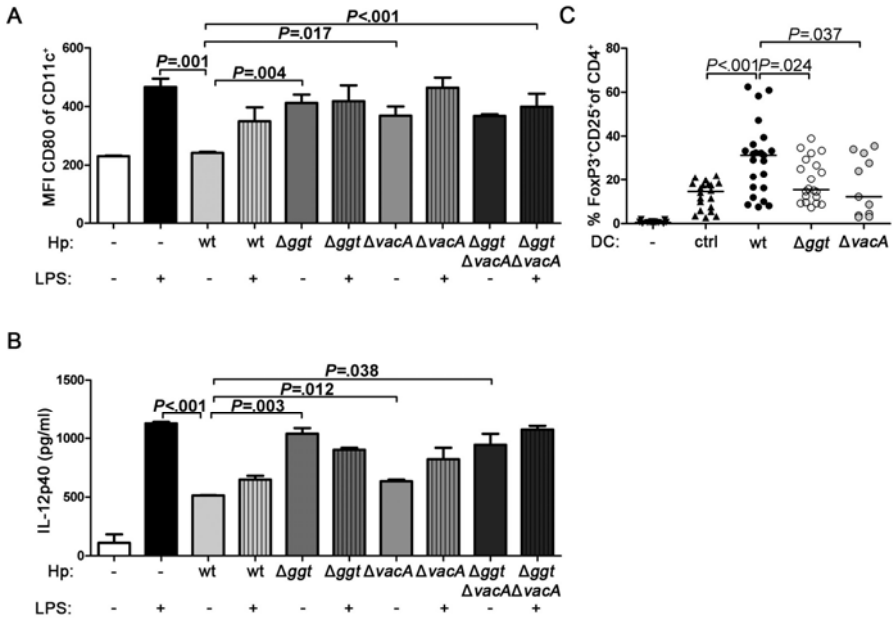


Fig. 11: Inhibition of BMDC maturation and tolerogenic re-programming by *H. pylori* depends on the virulence factors VacA and GGT. BMDCs were infected with either *H. pylori* wild type (wt) or isogenic mutants lacking the virulence factors VacA (Δ*vacA*) or GGT (Δ*ggt*). Flow cytometric analysis of CD80 expression (**A**). Quantification of IL-12p40 secretion as assessed by ELISA (**B**). Capacity of infected BMDCs to drive FoxP3 expression in naive T cells (**C**).

parental wild type strain (see appendix 7.1), suggesting that both mutants elicited more articulated pathogen-specific T-cell responses. With the decreased capacity of both mutants for the tolerogenic re-programming *in vitro* in mind, we assessed whether the three different strains differentially affected DC tolerogenicity *in vivo*. Indeed, DCs immunomagnetically isolated from single cell MLN preparations from wild type-infected mice exhibited strong tolerogenic activity, whereas efficient induction of FoxP3 and CD25 expression in co-cultured naive T-cells was not observed with DCs from either uninfected controls or with DCs from mice infected with the Δ*vacA* and Δ*ggt* mutants after 1 month of infection (Fig 12B). At the later the point (2 months post infection), seven out of ten Δ*ggt*-infected animals had cleared the infection, and the Δ*vacA*-infected mice still exhibited lower colonization levels than the wild type-infected counterparts. The lower bacterial burden in Δ*vacA*-infected mice was accompanied by severe inflammation

and strongly aggravated preneoplastic lesions as compared to controls infected with the parental wild type strain, whereas Δ*ggt*-infected mice (most of which had cleared the infection) exhibited only mild, remaining inflammatory infiltrates (Fig 12C).

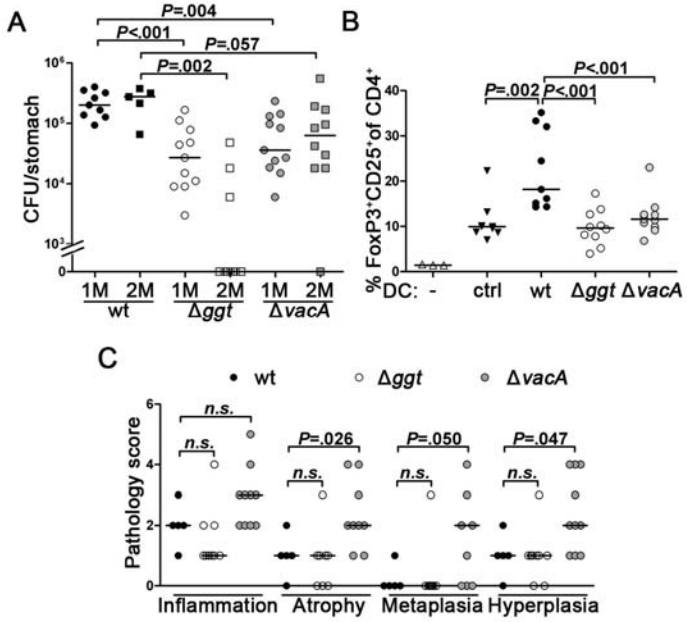


Fig. 12: Efficient gastric colonization by *H. pylori* and tolerogenic re-programming of DCs *in vivo* requires VacA and GGT. Gastric bacterial colonization after 1 month (1M) and 2 months (2M) of adult infection with either wild type *H. pylori* (wt) or the isogenic Δ*ggt* and Δ*vacA* mutants (**A**). Capacity of MLN DCs of uninfected (ctrl) and 1 month infected (wt, Δ*ggt* and Δ*vacA*) mice to induce FoxP3 and CD25 expression in naive T-cells (**B**). Pathology scores of mice shown in **A**, 2 months time point (**C**).

The defect of the Δ*ggt* mutant *in vitro* and *in vivo* to tolerize DCs was phenocopied by pharmacological inhibition of its enzymatic activity, suggesting that the transpeptidase activity of GGT is required for its immunomodulatory effects (see appendix 7.1). In conclusion, both VacA and GGT contribute to DC tolerization *in vitro* and *in vivo*, and it is tempting to speculate that this mechanism consequently leads to the more efficient clearance of the mutant bacteria relative to the wild type strain.

Our *in vivo* data are in line with the observation that VacA is not absolutely required for colonization, but VacA-proficiency does improve the process [(216)]. However, epidemiological data suggest that strains expressing high levels of VacA correlate with more severe disease, contradicting our results [(217)]. Additionally, VacA has been shown

to induce cell death via the disruption of mitochondrial membranes [218, 219]. This induction of cell death in gastric epithelial cell is beneficial for *H. pylori* as it releases nutrients, but a possible effect on gastric DCs would be detrimental in terms of tolerogenic re-programming. The discrepancy might be explained by different VacA concentrations or different VacA variants (toxic s1m1 versus non-toxic s2m2 variants), leading to different disease outcomes. While low VacA concentrations could be beneficial for the bacteria by tolerogenic re-programming of DCs and facilitated access to nutrients, large quantities of VacA could induce a pathogenic state by means of enhanced cell death, especially if the affected cell type is not only the gastric epithelial lining but additionally affects gastric "tolerogenic" DCs. Additionally, VacA has been identified as a key factor in the *H. pylori*-mediated inhibition of human T-cells [29, 30, 220] *in vitro*. VacA interferes with the IL-2 receptor signaling pathway at the level of the Ca^{2+}-calmodulin-dependent phosphatase calcineurin, thereby inhibiting T cell proliferation. As a consequence, the nuclear translocation of the transcription factor nuclear factor of activated T-cells (NFAT) is blocked, resulting in down-regulation of IL-2 gene transcription.

Similarly, Gerhard *et al.* provided evidence for a complementary mechanism for the inhibition of human T-cell proliferation. The authors demonstrated that *H. pylori* GGT arrests antigen-activated T-cells in the G1 phase of the cell cycle by interfering with G1 cyclin-dependent kinase activity. Deletion of the *ggt* gene abrogated the inhibitory effect of the bacteria on T-cell proliferation [221, 222]. Moreover, it has been known that GGT contributes critically to gastric colonization of mice [223]. The effects of VacA and GGT overlap in the inhibition of human T-cell proliferation *in vitro*, although these effects have not been shown yet to be relevant in murine models of infection. The more pronounced Th1-/Th17-responses, accompanied by similar (Δ*ggt*-infected mice) or more severe (Δ*vacA*-infected mice) immunopathology, observed after 2 months of infection with the isogenic mutant strains compared to wild type-infected mice, suggest that the described effects of both proteins on T-cell proliferation is not critical *in vivo*. Nevertheless, we tested the effect of the Δ*vacA* and Δ*ggt* strains on the tolerization of MLN DCs in the T cell-deficient TCR-$\beta^{-/-}$ background. As mentioned above, the tolerance promoting effects of VacA- and GGT-proficient *H. pylori* on DCs in the TCR-$\beta^{-/-}$ background were similar as in wild type mice. Interestingly, the Δ*vacA* mutant exhibited similar colonization levels (Fig 13A) in this set-up of neonatal infection, but the DCs of the Δ*vacA*-infected mice showed a clear deficiency in their ability to drive FoxP3 and CD25 expression in naive T-

cells in our co-culture system (Fig 13B). Surprisingly, although adult-infected animals initially

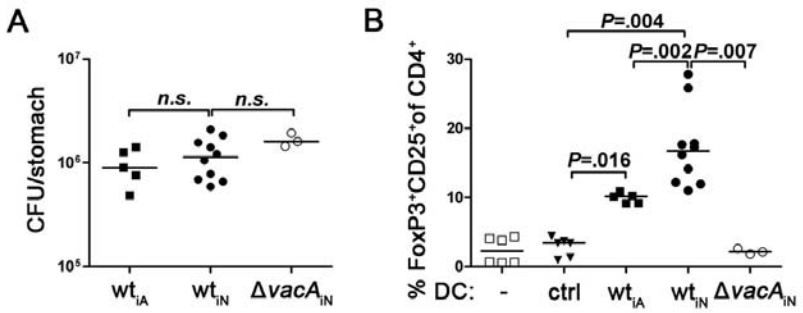


Fig. 13: The deficiency of the Δ*vacA* mutant in the re-programming of DCs is independent of T-cells in neonatal infection of TCR-β-/- mice. Colony forming units (CFU) of TCR-$\beta^{-/-}$ mice infected with *H. pylori* at 7 days (iN) or 6 weeks (iA) of age with either VacA-proficient (wt) or VacA-deficient (Δ*vacA*) *H. pylori* (**A**). Ability of MLN DCs isolated from **A** to convert naive T-cells to $FoxP3^+$ Tregs (**B**).

support colonization by the Δ*ggt* mutant, no Δ*ggt*-deficient bacteria could ever be retrieved from neonatally infected mice, independent of the T-cell proficiency of the host (data not shown and appendix). This difference in the ability of Δ*ggt* mutant strains to colonize adults versus their inability to settle in the juvenile stomach implies that GGT is crucial for initial colonization in early, neonatal infections and later on acts as a tolerance-promoting immunomodulator of the adult immune system.

The details of how VacA and GGT prevent DC maturation and promote DC tolerization need to be clarified yet. Most likely both factors act via entirely different mechanisms. In addition to the already mentioned effects of VacA on DC maturation, this virulence factor has been described to prevent autophagy in epithelial cells [(224)] and to interfere with phagosome maturation in macrophages [(225)]. If VacA acts in the same manner on DCs, their failure to respond properly to Δ*vacA* strains could be explained. With regard to the involvement of GGT, an explanation is less straight forward. Treatment of neonatally and adult mice infected with wild type *H. pylori* with acivicin, an inhibitor of GGT, either prevents colonization (neonatal infection) or reduces the bacterial burden (adult infection), suggesting that the effects depend on the enzymatic activity of GGT rather than on the molecule *per se*. Since GGT catalyzes the transfer of the gamma-glutamyl residue of glutathione or glutamine to amino acids or peptides generating cysteines, a

possible role of glutathione on DC tolerization can be considered and will be tested by supplementation with increasing concentrations of both compounds.

In conclusion, both bacterial factors VacA and GGT are critically involved in the re-programming of DCs towards a tolerance-promoting phenotype, thereby enabling initial and persistent infection of the host. Identifying such bacterial "tolerance factors" is of great importance and their putative beneficial effects on immune homeostasis might be used someday to develop either sterilizing and/or tolerizing vaccinations against *H. pylori*.

4.5 *HELICOBACTER PYLORI*-SPECIFIC TOLERANCE AND ASTHMA

As mentioned in section 1.2.3, several epidemiological surveys suggest an inverse association between *H. pylori* infections and asthma, as well as allergic and chronic inflammatory diseases [71-74, 215, 226]. This correlation is especially evident in children and young adults, which are less likely to carry the pathogen [71-73]. While infection rates has declined from >50% at the beginning of the 20th century to <10% at its end, the prevalence of early on-set asthma has increased at an astonishing rate in the same time period [226, 227] (Fig 4B). First experimental data to underscore an inverse correlation were provided by our lab. Arnold *et al.* found that neonatal infection with *H. pylori* protects mice against the clinical and histopathological symptoms of asthma, i.e. airway hyper-responsiveness, tissue inflammation, goblet cell metaplasia, and prevents the infiltration of eosinophils, Th2 and Th17 cells into the bronchoalveolar fluid. Asthma protection could be transferred from neonatally infected mice to challenged recipients via the transfer of relatively small numbers of purified Tregs [70].

Interestingly, the lungs of neonatally infected mice were infiltrated primarily with semi-mature DCs, whereas mature DCs constituted the majority of DCs in asthmatic lungs, suggesting a causal relationship between tolerance-promoting immune cell populations (Tregs and semi-mature DCs) and asthma protection [70]. Having observed the requirement for DC-derived IL-18 in Treg differentiation *in vitro* and *in vivo*, we assumed that Tregs from neonatally infected *IL-18*$^{-/-}$ *or IL-18r*$^{-/-}$ mice should lack this suppressive activity in the model of allergen-induced asthma. Indeed, Tregs isolated from neonatally infected wild type mice but not *IL-18*$^{-/-}$ *or IL-18r*$^{-/-}$ mice suppressed experimentally induced allergic airway disease, highlighting again the importance of IL-18 for Treg

differentiation *in vivo*. Using the same model, we were able to show that mice neonatally infected with *H. pylori* lacking VacA (Δ*vacA*) are not protected against the clinical and histopathological manifestations of disease, again underscoring the importance of VacA in the induction of functional, suppressive Tregs (see appendix 7.1). Since the Δ*ggt* mutant is not able to colonize its hosts in the neonatal period no data on the loss of protection in the asthma set-up could be gathered with GGT-deficient strains, but preliminary results suggest that intraperitoneal injection of 5µg/g bodyweight purified GGT to uninfected neonates over 3 weeks is sufficient to protect from allergen-induced asthma (unpublished data). This protective effect of the purified GGT supports the necessity for GGT-proficiency to induce *H. pylori*-specific immune tolerance. In the near

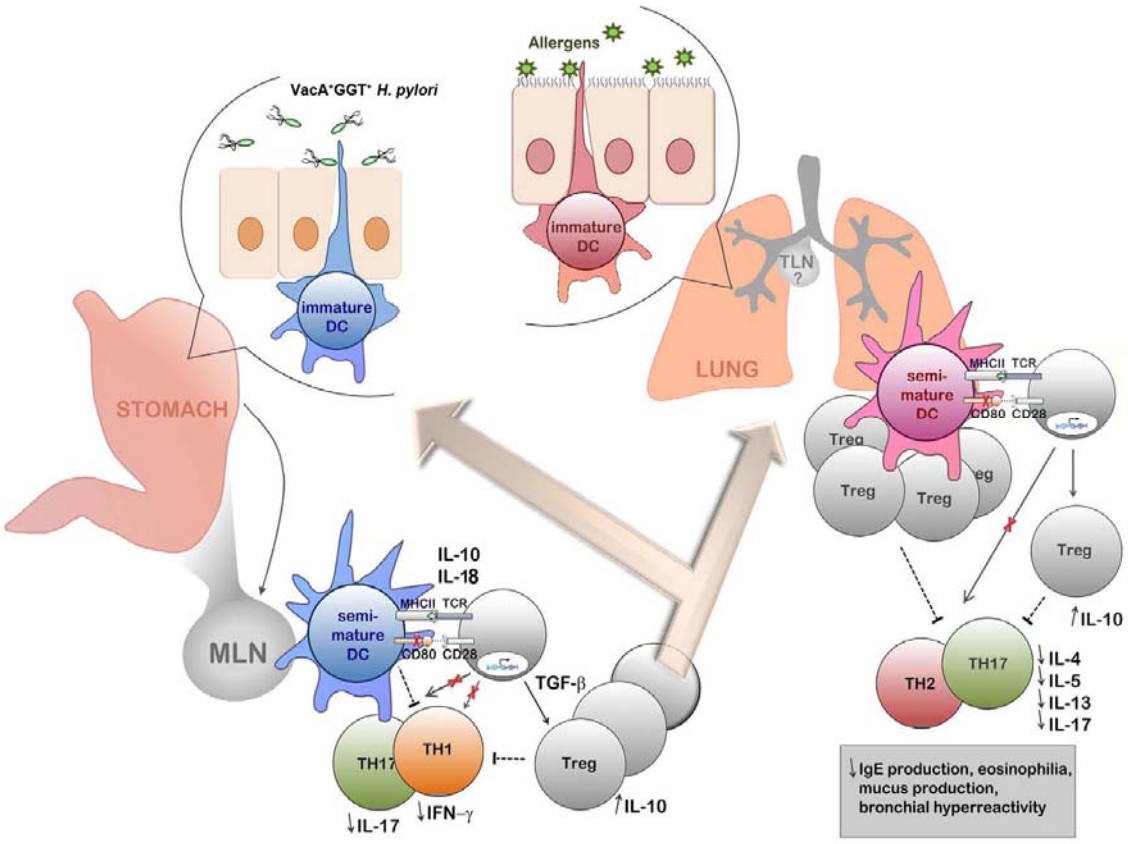


Fig. 14: Current working model of *H. pylori*-induced immune tolerance and asthma protection: In chronically infected hosts, VacA- and GGT-proficient *H. pylori* resides in the gastric mucosa where it is detected by tissue-resident DCs sampling the gastric lumen. *H. pylori*-experienced DCs acquire a semi-mature, tolerogenic phenotype prior to migration to the gut-draining MLN, where they induce CD25+FoxP3+ Tregs in an IL18- and TGF-β-dependent manner, but fail to effectively induce H. pylori-specific Th1-/Th17-responses. Peripherally induced Tregs prevent excessive gastric immunopathology and enable the bacteria

to persist in the host by retaining MLN DCs in a semi-mature state and by directly suppressing pathogen-specific Th1-/Th17-responses. Once induced, Treg migrate to the lungs where they suppress allergen-specific Th2-/Th17-responses, thereby preventing the pathogenesis of asthma. Prevention of allergic T-cell responses is either achieved by suppression of Th2- and Th17-responses by Treg/T-effector contact, secretion of soluble cytokines like IL-10, and/or by retaining lung-resident DCs in a semi-mature state. Suppression of Th2-/Th17-responses and the inhibition of DC maturation leads to reduced eosinophilia, mucus production, goblet cell metaplasia and airway hyper-responsiveness. Adapted from [215].

future it will be of great interest to elucidate receptors (NLR`s, C-type lectins?) and signaling pathways (wnt-, β-catenin signaling?) engaged by VacA and GGT in order to find the key to the tolerogenic re-programming of DCs by *H. pylori*. Although the initial modulation of the immune system towards tolerogenicity by the induction of semi-mature DCs likely takes place in the gastric mucosa, with the subsequent instruction of naive T cells to become Tregs in the draining MLNs, asthma protection is most likely not achieved by migratory gastric DCs infiltrating the lungs. It is more likely that newly induced Tregs migrate into the lungs, where they directly block allergic T cell responses by suppressing allergen-induced Th2-/Th17-polarization. The block of T cell responses can either be achieved via Treg/T-effector cell contact or via soluble cytokines, in particular IL-10. Alternatively, the migrated Tregs retain lung DCs in a semi-mature state, thereby preventing the instruction of allergen-specific T-cells and/or inducing $FoxP3^+$ Tregs in the tracheal lymph nodes (Fig. 14).

4.6. SYNTHESIS

The gram-negative bacterial pathogen *H. pylori* colonizes the gastric mucosa of one half of the world population. The infection is acquired in early childhood and typically persists for many decades. Humans and *H. pylori* have intimately co-existed at least since our ancestors first migrated out of East Africa 60000 years ago [228]. This long period of co-existence allowed the bacteria to aquire traits that enable them to evade immune surveillance by subverting both innate and adaptive components of the immune system to ensure persistence despite a vigorous, yet non sterilizing, local and systemic immune response. On the other hand, modulation of the immune system by *H. pylori* protects carriers against asthma and probably other allergic, chronic, inflammatory and

autoimmune diseases. The possible loss of fitness due to gastric pathology has been proposed to be offset by these protective effects against a variety of diseases.

The present work delivers some crucial components involved in mediating *H. pylori*-specific tolerance and protection against asthma. We were able to identify DCs, a critical link between the innate and adaptive branches of the immune system, as a fundamental part of this symbiosis. Moreover, we highlighted the necessity for different cytokines, i.e. IL-18 and TGF-β, to enable the bacteria to persist in its host and to confer asthma protection. Finally, we identified two bacterial factors, namely VacA and GGT, that induce tolerogenic DCs. These findings help us to understand the immunomodulatory properties of *H. pylori* and its putative beneficial effects on immune homeostasis, thereby opening up new avenues for the prevention of allergies and possible other chronic inflammatory diseases of increasing importance in industrialized countries from which *H. pylori* is disappearing.

5. REFERENCES

1. Marshall, B. 1983. Unidentified curved bacilli on gastric epithelium in active chronic gastritis. *Lancet* 1:1273-1275.
2. Marshall, B. J. 1986. Campylobacter pyloridis and gastritis. *J Infect Dis* 153:650-657.
3. Warrelmann, M., H. Menge, H. Schmidt, E. O. Riecken, and H. Hahn. 1987. Comparative investigations about three different identification systems for Campylobacter pyloridis from human antrum mucosa. *Zentralbl Bakteriol Mikrobiol Hyg A* 266:212-217.
4. Smet, A., B. Flahou, I. Mukhopadhya, R. Ducatelle, F. Pasmans, F. Haesebrouck, and G. L. Hold. The other Helicobacters. *Helicobacter* 16 Suppl 1:70-75.
5. Haesebrouck, F., F. Pasmans, B. Flahou, K. Chiers, M. Baele, T. Meyns, A. Decostere, and R. Ducatelle. 2009. Gastric helicobacters in domestic animals and nonhuman primates and their significance for human health. *Clin Microbiol Rev* 22:202-223, Table of Contents.
6. Owen, R. J. 1998. Helicobacter--species classification and identification. *Br Med Bull* 54:17-30.
7. Hazell, S. L., and A. Lee. 1986. Campylobacter pyloridis, urease, hydrogen ion back diffusion, and gastric ulcers. *Lancet* 2:15-17.
8. O'Toole, P. W., M. C. Lane, and S. Porwollik. 2000. Helicobacter pylori motility. *Microbes Infect* 2:1207-1214.
9. Leunk, R. D., P. T. Johnson, B. C. David, W. G. Kraft, and D. R. Morgan. 1988. Cytotoxic activity in broth-culture filtrates of Campylobacter pylori. *J Med Microbiol* 26:93-99.
10. Neman-Simha, V., and F. Megraud. 1988. In vitro model for Campylobacter pylori adherence properties. *Infect Immun* 56:3329-3333.
11. Basso, D., M. Plebani, and J. G. Kusters. Pathogenesis of Helicobacter pylori infection. *Helicobacter* 15 Suppl 1:14-20.
12. Mobley, H. L. T. 2001. Urease.
13. Andrutis, K. A., J. G. Fox, D. B. Schauer, R. P. Marini, J. C. Murphy, L. Yan, and J. V. Solnick. 1995. Inability of an isogenic urease-negative mutant stain of Helicobacter mustelae to colonize the ferret stomach. *Infect Immun* 63:3722-3725.
14. Eaton, K. A., C. L. Brooks, D. R. Morgan, and S. Krakowka. 1991. Essential role of urease in pathogenesis of gastritis induced by Helicobacter pylori in gnotobiotic piglets. *Infect Immun* 59:2470-2475.
15. Eaton, K. A., D. R. Morgan, and S. Krakowka. 1989. Campylobacter pylori virulence factors in gnotobiotic piglets. *Infect Immun* 57:1119-1125.
16. Eaton, K. A., D. R. Morgan, and S. Krakowka. 1992. Motility as a factor in the colonisation of gnotobiotic piglets by Helicobacter pylori. *J Med Microbiol* 37:123-127.
17. Eaton, K. A., S. Suerbaum, C. Josenhans, and S. Krakowka. 1996. Colonization of gnotobiotic piglets by Helicobacter pylori deficient in two flagellin genes. *Infect Immun* 64:2445-2448.
18. Stein, M., R. Rappuoli, and A. Covacci. 2001. The cag Pathogenicity Island.

19. Odenbreit, S., J. Puls, B. Sedlmaier, E. Gerland, W. Fischer, and R. Haas. 2000. Translocation of Helicobacter pylori CagA into gastric epithelial cells by type IV secretion. *Science* 287:1497-1500.
20. Stein, M., R. Rappuoli, and A. Covacci. 2000. Tyrosine phosphorylation of the Helicobacter pylori CagA antigen after cag-driven host cell translocation. *Proc Natl Acad Sci U S A* 97:1263-1268.
21. Segal, E. D., J. Cha, J. Lo, S. Falkow, and L. S. Tompkins. 1999. Altered states: involvement of phosphorylated CagA in the induction of host cellular growth changes by Helicobacter pylori. *Proc Natl Acad Sci U S A* 96:14559-14564.
22. Guillemin, K., N. R. Salama, L. S. Tompkins, and S. Falkow. 2002. Cag pathogenicity island-specific responses of gastric epithelial cells to Helicobacter pylori infection. *Proc Natl Acad Sci U S A* 99:15136-15141.
23. Sharma, S. A., M. K. Tummuru, M. J. Blaser, and L. D. Kerr. 1998. Activation of IL-8 gene expression by Helicobacter pylori is regulated by transcription factor nuclear factor-kappa B in gastric epithelial cells. *J Immunol* 160:2401-2407.
24. Viala, J., C. Chaput, I. G. Boneca, A. Cardona, S. E. Girardin, A. P. Moran, R. Athman, S. Memet, M. R. Huerre, A. J. Coyle, P. S. DiStefano, P. J. Sansonetti, A. Labigne, J. Bertin, D. J. Philpott, and R. L. Ferrero. 2004. Nod1 responds to peptidoglycan delivered by the Helicobacter pylori cag pathogenicity island. *Nat Immunol* 5:1166-1174.
25. Suzuki, M., H. Mimuro, K. Kiga, M. Fukumatsu, N. Ishijima, H. Morikawa, S. Nagai, S. Koyasu, R. H. Gilman, D. Kersulyte, D. E. Berg, and C. Sasakawa. 2009. Helicobacter pylori CagA phosphorylation-independent function in epithelial proliferation and inflammation. *Cell Host Microbe* 5:23-34.
26. Kusters, J. G., A. H. van Vliet, and E. J. Kuipers. 2006. Pathogenesis of Helicobacter pylori infection. *Clin Microbiol Rev* 19:449-490.
27. Cover, T. L., and S. R. Blanke. 2005. Helicobacter pylori VacA, a paradigm for toxin multifunctionality. *Nat Rev Microbiol* 3:320-332.
28. Molinari, M., M. Salio, C. Galli, N. Norais, R. Rappuoli, A. Lanzavecchia, and C. Montecucco. 1998. Selective inhibition of Ii-dependent antigen presentation by Helicobacter pylori toxin VacA. *J Exp Med* 187:135-140.
29. Sundrud, M. S., V. J. Torres, D. Unutmaz, and T. L. Cover. 2004. Inhibition of primary human T cell proliferation by Helicobacter pylori vacuolating toxin (VacA) is independent of VacA effects on IL-2 secretion. *Proc Natl Acad Sci U S A* 101:7727-7732.
30. Boncristiano, M., S. R. Paccani, S. Barone, C. Ulivieri, L. Patrussi, D. Ilver, A. Amedei, M. M. D'Elios, J. L. Telford, and C. T. Baldari. 2003. The Helicobacter pylori vacuolating toxin inhibits T cell activation by two independent mechanisms. *J Exp Med* 198:1887-1897.
31. Backert, S., and M. Clyne. Pathogenesis of Helicobacter pylori infection. *Helicobacter* 16 Suppl 1:19-25.
32. Carlsohn, E., J. Nystrom, I. Bolin, C. L. Nilsson, and A. M. Svennerholm. 2006. HpaA is essential for Helicobacter pylori colonization in mice. *Infect Immun* 74:920-926.
33. Satin, B., G. Del Giudice, V. Della Bianca, S. Dusi, C. Laudanna, F. Tonello, D. Kelleher, R. Rappuoli, C. Montecucco, and F. Rossi. 2000. The neutrophil-activating protein (HP-NAP) of Helicobacter pylori is a protective antigen and a major virulence factor. *J Exp Med* 191:1467-1476.
34. Wang, G., Y. Hong, A. Olczak, S. E. Maier, and R. J. Maier. 2006. Dual Roles of Helicobacter pylori NapA in inducing and combating oxidative stress. *Infect Immun* 74:6839-6846.

35. Gong, M., S. S. Ling, S. Y. Lui, K. G. Yeoh, and B. Ho. 2010. Helicobacter pylori gamma-glutamyl transpeptidase is a pathogenic factor in the development of peptic ulcer disease. *Gastroenterology* 139:564-573.
36. Kim, K. M., S. G. Lee, J. M. Kim, D. S. Kim, J. Y. Song, H. L. Kang, W. K. Lee, M. J. Cho, K. H. Rhee, H. S. Youn, and S. C. Baik. Helicobacter pylori gamma-glutamyltranspeptidase induces cell cycle arrest at the G1-S phase transition. *J Microbiol* 48:372-377.
37. Olbermann, P., C. Josenhans, Y. Moodley, M. Uhr, C. Stamer, M. Vauterin, S. Suerbaum, M. Achtman, and B. Linz. A global overview of the genetic and functional diversity in the Helicobacter pylori cag pathogenicity island. *PLoS Genet* 6:e1001069.
38. Lee, Y. C., J. M. Liou, M. S. Wu, C. Y. Wu, and J. T. Lin. 2008. Eradication of helicobacter pylori to prevent gastroduodenal diseases: hitting more than one bird with the same stone. *Therap Adv Gastroenterol* 1:111-120.
39. Brown, L. M. 2000. Helicobacter pylori: epidemiology and routes of transmission. *Epidemiol Rev* 22:283-297.
40. Granstrom, M., Y. Tindberg, and M. Blennow. 1997. Seroepidemiology of Helicobacter pylori infection in a cohort of children monitored from 6 months to 11 years of age. *J Clin Microbiol* 35:468-470.
41. Rothenbacher, D., J. Inceoglu, G. Bode, and H. Brenner. 2000. Acquisition of Helicobacter pylori infection in a high-risk population occurs within the first 2 years of life. *J Pediatr* 136:744-748.
42. Bauer. 2011. The human gastric pathogen *Helicobacter pylori* and its association with gastric cancer and ulcer disease. *Ulcers 2011:23*.
43. Ernst, P. B., and B. D. Gold. 2000. The disease spectrum of Helicobacter pylori: the immunopathogenesis of gastroduodenal ulcer and gastric cancer. *Annu Rev Microbiol* 54:615-640.
44. Peek, R. M., Jr., and J. E. Crabtree. 2006. Helicobacter infection and gastric neoplasia. *J Pathol* 208:233-248.
45. Parsonnet, J., S. Hansen, L. Rodriguez, A. B. Gelb, R. A. Warnke, E. Jellum, N. Orentreich, J. H. Vogelman, and G. D. Friedman. 1994. Helicobacter pylori infection and gastric lymphoma. *N Engl J Med* 330:1267-1271.
46. Jemal, A., F. Bray, M. M. Center, J. Ferlay, E. Ward, and D. Forman. 2011. Global cancer statistics. *CA Cancer J Clin* 61:69-90.
47. Correa, P., and J. Houghton. 2007. Carcinogenesis of Helicobacter pylori. *Gastroenterology* 133:659-672.
48. Houghton, J., and T. C. Wang. 2005. Helicobacter pylori and gastric cancer: a new paradigm for inflammation-associated epithelial cancers. *Gastroenterology* 128:1567-1578.
49. Eck, M., B. Schmausser, R. Haas, A. Greiner, S. Czub, and H. K. Muller-Hermelink. 1997. MALT-type lymphoma of the stomach is associated with Helicobacter pylori strains expressing the CagA protein. *Gastroenterology* 112:1482-1486.
50. Wotherspoon, A. C., C. Ortiz-Hidalgo, M. R. Falzon, and P. G. Isaacson. 1991. Helicobacter pylori-associated gastritis and primary B-cell gastric lymphoma. *Lancet* 338:1175-1176.
51. Hatakeyama, M. 2008. [Oncogenic mechanism of Helicobacter pylori]. *Nihon Rinsho Meneki Gakkai Kaishi* 31:132-140.
52. Ohnishi, N., H. Yuasa, S. Tanaka, H. Sawa, M. Miura, A. Matsui, H. Higashi, M. Musashi, K. Iwabuchi, M. Suzuki, G. Yamada, T. Azuma, and M. Hatakeyama. 2008. Transgenic expression of Helicobacter pylori CagA induces gastrointestinal

and hematopoietic neoplasms in mouse. *Proc Natl Acad Sci U S A* 105:1003-1008.

53. El-Omar, E. M., M. Carrington, W. H. Chow, K. E. McColl, J. H. Bream, H. A. Young, J. Herrera, J. Lissowska, C. C. Yuan, N. Rothman, G. Lanyon, M. Martin, J. F. Fraumeni, Jr., and C. S. Rabkin. 2000. Interleukin-1 polymorphisms associated with increased risk of gastric cancer. *Nature* 404:398-402.
54. Tu, S., G. Bhagat, G. Cui, S. Takaishi, E. A. Kurt-Jones, B. Rickman, K. S. Betz, M. Penz-Oesterreicher, O. Bjorkdahl, J. G. Fox, and T. C. Wang. 2008. Overexpression of interleukin-1beta induces gastric inflammation and cancer and mobilizes myeloid-derived suppressor cells in mice. *Cancer Cell* 14:408-419.
55. Lauwers, G. Y. 2003. Defining the pathologic diagnosis of metaplasia, atrophy, dysplasia, and gastric adenocarcinoma. *J Clin Gastroenterol* 36:S37-43; discussion S61-32.
56. Peek, R. M., Jr., C. Fiske, and K. T. Wilson. 2009. Role of innate immunity in Helicobacter pylori-induced gastric malignancy. *Physiol Rev* 90:831-858.
57. Rogers, A. B., N. S. Taylor, M. T. Whary, E. D. Stefanich, T. C. Wang, and J. G. Fox. 2005. Helicobacter pylori but not high salt induces gastric intraepithelial neoplasia in B6129 mice. *Cancer Res* 65:10709-10715.
58. Toller, I. M., K. J. Neelsen, M. Steger, M. L. Hartung, M. O. Hottiger, M. Stucki, B. Kalali, M. Gerhard, A. A. Sartori, M. Lopes, and A. Muller. Carcinogenic bacterial pathogen Helicobacter pylori triggers DNA double-strand breaks and a DNA damage response in its host cells. *Proc Natl Acad Sci U S A* 108:14944-14949.
59. Uemura, N., T. Mukai, S. Okamoto, S. Yamaguchi, H. Mashiba, K. Taniyama, N. Sasaki, K. Haruma, K. Sumii, and G. Kajiyama. 1997. Effect of Helicobacter pylori eradication on subsequent development of cancer after endoscopic resection of early gastric cancer. *Cancer Epidemiol Biomarkers Prev* 6:639-642.
60. Maehata, Y., S. Nakamura, K. Fujisawa, M. Esaki, T. Moriyama, K. Asano, Y. Fuyuno, K. Yamaguchi, I. Egashira, H. Kim, M. Kanda, M. Hirahashi, and T. Matsumoto. 2012. Long-term effect of Helicobacter pylori eradication on the development of metachronous gastric cancer after endoscopic resection of early gastric cancer. *Gastrointest Endosc* 75:39-46.
61. Vannella, L., E. Lahner, C. Bordi, E. Pilozzi, E. Di Giulio, V. D. Corleto, J. Osborn, G. Delle Fave, and B. Annibale. 2012. Reversal of atrophic body gastritis after H. pylori eradication at long-term follow-up. *Dig Liver Dis* 43:295-299.
62. Talley, N. J. 2005. American Gastroenterological Association medical position statement: evaluation of dyspepsia. *Gastroenterology* 129:1753-1755.
63. Corley, D. A., A. Kubo, T. R. Levin, G. Block, L. Habel, G. Rumore, C. Quesenberry, P. Buffler, and J. Parsonnet. 2008. Helicobacter pylori and gastroesophageal reflux disease: a case-control study. *Helicobacter* 13:352-360.
64. Huang, B., Y. Chen, Q. Xie, G. Lin, Y. Wu, Y. Feng, J. Li, Y. Zhuo, and P. Zhang. CagA-positive Helicobacter pylori strains enhanced coronary atherosclerosis by increasing serum OxLDL and HsCRP in patients with coronary heart disease. *Dig Dis Sci* 56:109-114.
65. Niccoli, G., F. Franceschi, N. Cosentino, B. Giupponi, G. De Marco, G. Merra, M. Conte, R. A. Montone, G. Ferrante, M. Baca, A. Gasbarrini, N. G. Silveri, and F. Crea. Coronary atherosclerotic burden in patients with infection by CagA-positive strains of Helicobacter pylori. *Coron Artery Dis* 21:217-221.
66. Schimke, K., S. A. Chubb, W. A. Davis, and T. M. Davis. Helicobacter pylori cytotoxin-associated gene-A antibodies do not predict complications or death in type 2 diabetes: the Fremantle Diabetes Study. *Atherosclerosis* 212:321-326.

67. Milman, N., S. Rosenstock, L. Andersen, T. Jorgensen, and O. Bonnevie. 1998. Serum ferritin, hemoglobin, and Helicobacter pylori infection: a seroepidemiologic survey comprising 2794 Danish adults. *Gastroenterology* 115:268-274.
68. Stasi, R., A. Sarpatwari, J. B. Segal, J. Osborn, M. L. Evangelista, N. Cooper, D. Provan, A. Newland, S. Amadori, and J. B. Bussel. 2009. Effects of eradication of Helicobacter pylori infection in patients with immune thrombocytopenic purpura: a systematic review. *Blood* 113:1231-1240.
69. Suzuki, H., F. Franceschi, T. Nishizawa, and A. Gasbarrini. 2011. Extragastric manifestations of Helicobacter pylori infection. *Helicobacter* 16 Suppl 1:65-69.
70. Arnold, I. C., N. Dehzad, S. Reuter, H. Martin, B. Becher, C. Taube, and A. Muller. 2011. Helicobacter pylori infection prevents allergic asthma in mouse models through the induction of regulatory T cells. *J Clin Invest* 121:3088-3093.
71. Blaser, M. J., Y. Chen, and J. Reibman. 2008. Does Helicobacter pylori protect against asthma and allergy? *Gut* 57:561-567.
72. Chen, Y., and M. J. Blaser. 2008. Helicobacter pylori colonization is inversely associated with childhood asthma. *J Infect Dis* 198:553-560.
73. Reibman, J., M. Marmor, J. Filner, M. E. Fernandez-Beros, L. Rogers, G. I. Perez-Perez, and M. J. Blaser. 2008. Asthma is inversely associated with Helicobacter pylori status in an urban population. *PLoS One* 3:e4060.
74. Amberbir, A., G. Medhin, W. Erku, A. Alem, R. Simms, K. Robinson, A. Fogarty, J. Britton, A. Venn, and G. Davey. 2011. Effects of Helicobacter pylori, geohelminth infection and selected commensal bacteria on the risk of allergic disease and sensitization in 3-year-old Ethiopian children. *Clin Exp Allergy* 41:1422-1430.
75. Rook, G. A. 2009. 99th Dahlem conference on infection, inflammation and chronic inflammatory disorders: darwinian medicine and the 'hygiene' or 'old friends' hypothesis. *Clin Exp Immunol* 160:70-79.
76. Masoli. 2010. Global burden of asthma.
77. Kumar Pachathundikandi, S., S. Brandt, J. Madassery, and S. Backert. 2010. Induction of TLR-2 and TLR-5 expression by Helicobacter pylori switches cagPAI-dependent signalling leading to the secretion of IL-8 and TNF-alpha. *PLoS One* 6:e19614.
78. Smith, M. F., Jr., A. Mitchell, G. Li, S. Ding, A. M. Fitzmaurice, K. Ryan, S. Crowe, and J. B. Goldberg. 2003. Toll-like receptor (TLR) 2 and TLR5, but not TLR4, are required for Helicobacter pylori-induced NF-kappa B activation and chemokine expression by epithelial cells. *J Biol Chem* 278:32552-32560.
79. Allison, C. C., T. A. Kufer, E. Kremmer, M. Kaparakis, and R. L. Ferrero. 2009. Helicobacter pylori induces MAPK phosphorylation and AP-1 activation via a NOD1-dependent mechanism. *J Immunol* 183:8099-8109.
80. Backhed, F., B. Rokbi, E. Torstensson, Y. Zhao, C. Nilsson, D. Seguin, S. Normark, A. M. Buchan, and A. Richter-Dahlfors. 2003. Gastric mucosal recognition of Helicobacter pylori is independent of Toll-like receptor 4. *J Infect Dis* 187:829-836.
81. Bodger, K., and J. E. Crabtree. 1998. Helicobacter pylori and gastric inflammation. *Br Med Bull* 54:139-150.
82. Maeda, S., M. Akanuma, Y. Mitsuno, Y. Hirata, K. Ogura, H. Yoshida, Y. Shiratori, and M. Omata. 2001. Distinct mechanism of Helicobacter pylori-mediated NF-kappa B activation between gastric cancer cells and monocytic cells. *J Biol Chem* 276:44856-44864.

83. Kawahara, T., Y. Kuwano, S. Teshima-Kondo, T. Kawai, T. Nikawa, K. Kishi, and K. Rokutan. 2001. Toll-like receptor 4 regulates gastric pit cell responses to Helicobacter pylori infection. *J Med Invest* 48:190-197.
84. Su, B., P. J. Ceponis, S. Lebel, H. Huynh, and P. M. Sherman. 2003. Helicobacter pylori activates Toll-like receptor 4 expression in gastrointestinal epithelial cells. *Infect Immun* 71:3496-3502.
85. Lee, S. K., A. Stack, E. Katzowitsch, S. I. Aizawa, S. Suerbaum, and C. Josenhans. 2003. Helicobacter pylori flagellins have very low intrinsic activity to stimulate human gastric epithelial cells via TLR5. *Microbes Infect* 5:1345-1356.
86. Sobala, G. M., J. E. Crabtree, M. F. Dixon, C. J. Schorah, J. D. Taylor, B. J. Rathbone, R. V. Heatley, and A. T. Axon. 1991. Acute Helicobacter pylori infection: clinical features, local and systemic immune response, gastric mucosal histology, and gastric juice ascorbic acid concentrations. *Gut* 32:1415-1418.
87. Obonyo, M., M. Sabet, S. P. Cole, J. Ebmeyer, S. Uematsu, S. Akira, and D. G. Guiney. 2007. Deficiencies of myeloid differentiation factor 88, Toll-like receptor 2 (TLR2), or TLR4 produce specific defects in macrophage cytokine secretion induced by Helicobacter pylori. *Infect Immun* 75:2408-2414.
88. Sayi, A., E. Kohler, I. M. Toller, R. A. Flavell, W. Muller, A. Roers, and A. Muller. 2011. TLR-2-activated B cells suppress Helicobacter-induced preneoplastic gastric immunopathology by inducing T regulatory-1 cells. *J Immunol* 186:878-890.
89. Necchi, V., R. Manca, V. Ricci, and E. Solcia. 2009. Evidence for transepithelial dendritic cells in human H. pylori active gastritis. *Helicobacter* 14:208-222.
90. Niess, J. H., S. Brand, X. Gu, L. Landsman, S. Jung, B. A. McCormick, J. M. Vyas, M. Boes, H. L. Ploegh, J. G. Fox, D. R. Littman, and H. C. Reinecker. 2005. CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. *Science* 307:254-258.
91. Rescigno, M., G. Rotta, B. Valzasina, and P. Ricciardi-Castagnoli. 2001. Dendritic cells shuttle microbes across gut epithelial monolayers. *Immunobiology* 204:572-581.
92. Banchereau, J., F. Briere, C. Caux, J. Davoust, S. Lebecque, Y. J. Liu, B. Pulendran, and K. Palucka. 2000. Immunobiology of dendritic cells. *Annu Rev Immunol* 18:767-811.
93. Guiney, D. G., P. Hasegawa, and S. P. Cole. 2003. Helicobacter pylori preferentially induces interleukin 12 (IL-12) rather than IL-6 or IL-10 in human dendritic cells. *Infect Immun* 71:4163-4166.
94. Hafsi, N., P. Voland, S. Schwendy, R. Rad, W. Reindl, M. Gerhard, and C. Prinz. 2004. Human dendritic cells respond to Helicobacter pylori, promoting NK cell and Th1-effector responses in vitro. *J Immunol* 173:1249-1257.
95. Bergman, M. P., A. Engering, H. H. Smits, S. J. van Vliet, A. A. van Bodegraven, H. P. Wirth, M. L. Kapsenberg, C. M. Vandenbroucke-Grauls, Y. van Kooyk, and B. J. Appelmelk. 2004. Helicobacter pylori modulates the T helper cell 1/T helper cell 2 balance through phase-variable interaction between lipopolysaccharide and DC-SIGN. *J Exp Med* 200:979-990.
96. Gringhuis, S. I., J. den Dunnen, M. Litjens, M. van der Vlist, and T. B. Geijtenbeek. 2009. Carbohydrate-specific signaling through the DC-SIGN signalosome tailors immunity to Mycobacterium tuberculosis, HIV-1 and Helicobacter pylori. *Nat Immunol* 10:1081-1088.
97. Rad, R., W. Ballhorn, P. Voland, K. Eisenacher, J. Mages, L. Rad, R. Ferstl, R. Lang, H. Wagner, R. M. Schmid, S. Bauer, C. Prinz, C. J. Kirschning, and A. Krug. 2009. Extracellular and intracellular pattern recognition receptors

cooperate in the recognition of Helicobacter pylori. *Gastroenterology* 136:2247-2257.
98. Kao, J. Y., S. Rathinavelu, K. A. Eaton, L. Bai, Y. Zavros, M. Takami, A. Pierzchala, and J. L. Merchant. 2006. Helicobacter pylori-secreted factors inhibit dendritic cell IL-12 secretion: a mechanism of ineffective host defense. *Am J Physiol Gastrointest Liver Physiol* 291:G73-81.
99. DeLyria, E. S., R. W. Redline, and T. G. Blanchard. 2009. Vaccination of mice against H pylori induces a strong Th-17 response and immunity that is neutrophil dependent. *Gastroenterology* 136:247-256.
100. Mangan, P. R., L. E. Harrington, D. B. O'Quinn, W. S. Helms, D. C. Bullard, C. O. Elson, R. D. Hatton, S. M. Wahl, T. R. Schoeb, and C. T. Weaver. 2006. Transforming growth factor-beta induces development of the T(H)17 lineage. *Nature* 441:231-234.
101. Mariathasan, S., and D. M. Monack. 2007. Inflammasome adaptors and sensors: intracellular regulators of infection and inflammation. *Nat Rev Immunol* 7:31-40.
102. D'Elios, M. M., M. Manghetti, F. Almerigogna, A. Amedei, F. Costa, D. Burroni, C. T. Baldari, S. Romagnani, J. L. Telford, and G. Del Prete. 1997. Different cytokine profile and antigen-specificity repertoire in Helicobacter pylori-specific T cell clones from the antrum of chronic gastritis patients with or without peptic ulcer. *Eur J Immunol* 27:1751-1755.
103. Eaton. 2001. The role of T cell subsets and cytokines in the pathogenesis of *Helicobacter pylori* gastritis in mice. *Journal of Immunology*:7456-7461.
104. Smythies, L. E., K. B. Waites, J. R. Lindsey, P. R. Harris, P. Ghiara, and P. D. Smith. 2000. Helicobacter pylori-induced mucosal inflammation is Th1 mediated and exacerbated in IL-4, but not IFN-gamma, gene-deficient mice. *J Immunol* 165:1022-1029.
105. Ermak TH, G. P., Nichols R, Myers GA, Nedrud J, Weltzin R, Lee CK, Kleanthous H, Monath TP. 1998. Immunization of mice with urease vaccine affords protection against Helicobacter pylori infection in the absence of antibodies and is mediated by MHC class II-restricted responses. *J Exp Med* 188:2277-2288.
106. Bamford, K. B., X. Fan, S. E. Crowe, J. F. Leary, W. K. Gourley, G. K. Luthra, E. G. Brooks, D. Y. Graham, V. E. Reyes, and P. B. Ernst. 1998. Lymphocytes in the human gastric mucosa during Helicobacter pylori have a T helper cell 1 phenotype. *Gastroenterology* 114:482-492.
107. Quiding-Jarbrink, M., B. S. Lundin, H. Lonroth, and A. M. Svennerholm. 2001. CD4+ and CD8+ T cell responses in Helicobacter pylori-infected individuals. *Clin Exp Immunol* 123:81-87.
108. Tan, M. P., J. Pedersen, Y. Zhan, A. M. Lew, M. J. Pearse, O. L. Wijburg, and R. A. Strugnell. 2008. CD8+ T cells are associated with severe gastritis in Helicobacter pylori-infected mice in the absence of CD4+ T cells. *Infect Immun* 76:1289-1297.
109. Mohammadi, M., S. Czinn, R. Redline, and J. Nedrud. 1996. Helicobacter-specific cell-mediated immune responses display a predominant Th1 phenotype and promote a delayed-type hypersensitivity response in the stomachs of mice. *J Immunol* 156:4729-4738.
110. Mohammadi M, N. J., Redline R, Lycke N, Czinn SJ. 1997. Murine CD4 T-cell response to *Helicobacter* infection: TH1 cells enhance gastritis and TH2 cells reduce bacterial load. *Annu Rev Immunol* 113:1848-1857.
111. Iwakura, Y., and H. Ishigame. 2006. The IL-23/IL-17 axis in inflammation. *J Clin Invest* 116:1218-1222.

112. Khamri, W., M. M. Walker, P. Clark, J. C. Atherton, M. R. Thursz, K. B. Bamford, R. I. Lechler, and G. Lombardi. Helicobacter pylori stimulates dendritic cells to induce interleukin-17 expression from CD4+ T lymphocytes. *Infect Immun* 78:845-853.
113. Luzza, F., T. Parrello, G. Monteleone, L. Sebkova, M. Romano, R. Zarrilli, M. Imeneo, and F. Pallone. 2000. Up-regulation of IL-17 is associated with bioactive IL-8 expression in Helicobacter pylori-infected human gastric mucosa. *J Immunol* 165:5332-5337.
114. Wilson, K. T., and J. E. Crabtree. 2007. Immunology of Helicobacter pylori: insights into the failure of the immune response and perspectives on vaccine studies. *Gastroenterology* 133:288-308.
115. Shi, Y., X. F. Liu, Y. Zhuang, J. Y. Zhang, T. Liu, Z. Yin, C. Wu, X. H. Mao, K. R. Jia, F. J. Wang, H. Guo, R. A. Flavell, Z. Zhao, K. Y. Liu, B. Xiao, Y. Guo, W. J. Zhang, W. Y. Zhou, G. Guo, and Q. M. Zou. 2011. Helicobacter pylori-induced Th17 responses modulate Th1 cell responses, benefit bacterial growth, and contribute to pathology in mice. *J Immunol* 184:5121-5129.
116. Shiomi, S., A. Toriie, S. Imamura, H. Konishi, S. Mitsufuji, Y. Iwakura, Y. Yamaoka, H. Ota, T. Yamamoto, J. Imanishi, and M. Kita. 2008. IL-17 is involved in Helicobacter pylori-induced gastric inflammatory responses in a mouse model. *Helicobacter* 13:518-524.
117. Kao, J. Y., M. Zhang, M. J. Miller, J. C. Mills, B. Wang, M. Liu, K. A. Eaton, W. Zou, B. E. Berndt, T. S. Cole, T. Takeuchi, S. Y. Owyang, and J. Luther. 2009. Helicobacter pylori immune escape is mediated by dendritic cell-induced Treg skewing and Th17 suppression in mice. *Gastroenterology* 138:1046-1054.
118. Rad, R., L. Brenner, S. Bauer, S. Schwendy, L. Layland, C. P. da Costa, W. Reindl, A. Dossumbekova, M. Friedrich, D. Saur, H. Wagner, R. M. Schmid, and C. Prinz. 2006. CD25+/Foxp3+ T cells regulate gastric inflammation and Helicobacter pylori colonization in vivo. *Gastroenterology* 131:525-537.
119. Raghavan, S., M. Fredriksson, A. M. Svennerholm, J. Holmgren, and E. Suri-Payer. 2003. Absence of CD4+CD25+ regulatory T cells is associated with a loss of regulation leading to increased pathology in Helicobacter pylori-infected mice. *Clin Exp Immunol* 132:393-400.
120. Freire de Melo, F., A. M. Rocha, G. A. Rocha, S. H. Pedroso, S. de Assis Batista, L. P. Fonseca de Castro, S. D. Carvalho, P. F. Bittencourt, C. A. de Oliveira, R. Correa-Oliveira, and D. M. Magalhaes Queiroz. A regulatory instead of an IL-17 T response predominates in Helicobacter pylori-associated gastritis in children. *Microbes Infect* 14:341-347.
121. Harris, P. R., S. W. Wright, C. Serrano, F. Riera, I. Duarte, J. Torres, A. Pena, A. Rollan, P. Viviani, E. Guiraldes, J. M. Schmitz, R. G. Lorenz, L. Novak, L. E. Smythies, and P. D. Smith. 2008. Helicobacter pylori gastritis in children is associated with a regulatory T-cell response. *Gastroenterology* 134:491-499.
122. Arnold, I. C., J. Y. Lee, M. R. Amieva, A. Roers, R. A. Flavell, T. Sparwasser, and A. Muller. 2011. Tolerance rather than immunity protects from Helicobacter pylori-induced gastric preneoplasia. *Gastroenterology* 140:199-209.
123. Robinson, K., R. Kenefeck, E. L. Pidgeon, S. Shakib, S. Patel, R. J. Polson, A. M. Zaitoun, and J. C. Atherton. 2008. Helicobacter pylori-induced peptic ulcer disease is associated with inadequate regulatory T cell responses. *Gut* 57:1375-1385.
124. Velin, D., L. Favre, E. Bernasconi, D. Bachmann, C. Pythoud, E. Saiji, H. Bouzourene, and P. Michetti. 2009. Interleukin-17 is a critical mediator of

vaccine-induced reduction of Helicobacter infection in the mouse model. *Gastroenterology* 136:2237-2246 e2231.
125. Cheng, H. H., G. Y. Tseng, H. B. Yang, H. J. Wang, H. J. Lin, and W. C. Wang. 2011. Increased numbers of Foxp3-positive regulatory T cells in gastritis, peptic ulcer and gastric adenocarcinoma. *World J Gastroenterol* 18:34-43.
126. Marshall, B. J., and H. M. Windsor. 2005. The relation of Helicobacter pylori to gastric adenocarcinoma and lymphoma: pathophysiology, epidemiology, screening, clinical presentation, treatment, and prevention. *Med Clin North Am* 89:313-344, viii.
127. Martin-Gayo, E., E. Sierra-Filardi, A. L. Corbi, and M. L. Toribio. 2010. Plasmacytoid dendritic cells resident in human thymus drive natural Treg cell development. *Blood* 115:5366-5375.
128. O'Keeffe, J., and A. P. Moran. 2008. Conventional, regulatory, and unconventional T cells in the immunologic response to Helicobacter pylori. *Helicobacter* 13:1-19.
129. Sprent, J., and C. D. Surh. 2003. Knowing one's self: central tolerance revisited. *Nat Immunol* 4:303-304.
130. Tsuji, N. M., and A. Kosaka. 2008. Oral tolerance: intestinal homeostasis and antigen-specific regulatory T cells. *Trends Immunol* 29:532-540.
131. Steinman, R. M., D. Hawiger, and M. C. Nussenzweig. 2003. Tolerogenic dendritic cells. *Annu Rev Immunol* 21:685-711.
132. Barton, G. M., and R. Medzhitov. 2003. Toll-like receptor signaling pathways. *Science* 300:1524-1525.
133. Franchi, L., R. Munoz-Planillo, and G. Nunez. 2010. Sensing and reacting to microbes through the inflammasomes. *Nat Immunol* 13:325-332.
134. Re, F., and J. L. Strominger. 2004. Heterogeneity of TLR-induced responses in dendritic cells: from innate to adaptive immunity. *Immunobiology* 209:191-198.
135. Barton, G. M., and R. Medzhitov. 2003. Linking Toll-like receptors to IFN-alpha/beta expression. *Nat Immunol* 4:432-433.
136. Alvarez, D., E. H. Vollmann, and U. H. von Andrian. 2008. Mechanisms and consequences of dendritic cell migration. *Immunity* 29:325-342.
137. Cronin, S. J., and J. M. Penninger. 2007. From T-cell activation signals to signaling control of anti-cancer immunity. *Immunol Rev* 220:151-168.
138. Meylan, E., and J. Tschopp. 2006. Toll-like receptors and RNA helicases: two parallel ways to trigger antiviral responses. *Mol Cell* 22:561-569.
139. Salter, R. D., and S. C. Watkins. 2009. Dendritic cell altered states: what role for calcium? *Immunol Rev* 231:278-288.
140. Proietto, A. I., S. van Dommelen, P. Zhou, A. Rizzitelli, A. D'Amico, R. J. Steptoe, S. H. Naik, M. H. Lahoud, Y. Liu, P. Zheng, K. Shortman, and L. Wu. 2008. Dendritic cells in the thymus contribute to T-regulatory cell induction. *Proc Natl Acad Sci U S A* 105:19869-19874.
141. Liu, Y. J., V. Soumelis, N. Watanabe, T. Ito, Y. H. Wang, W. Malefyt Rde, M. Omori, B. Zhou, and S. F. Ziegler. 2007. TSLP: an epithelial cell cytokine that regulates T cell differentiation by conditioning dendritic cell maturation. *Annu Rev Immunol* 25:193-219.
142. Wang, J., and F. Xing. 2008. Human TSLP-educated DCs. *Cell Mol Immunol* 5:99-106.
143. Watanabe, N., Y. H. Wang, H. K. Lee, T. Ito, W. Cao, and Y. J. Liu. 2005. Hassall's corpuscles instruct dendritic cells to induce CD4+CD25+ regulatory T cells in human thymus. *Nature* 436:1181-1185.

144. Chieppa, M., M. Rescigno, A. Y. Huang, and R. N. Germain. 2006. Dynamic imaging of dendritic cell extension into the small bowel lumen in response to epithelial cell TLR engagement. *J Exp Med* 203:2841-2852.
145. Hultkrantz, S., S. Ostman, and E. Telemo. 2005. Induction of antigen-specific regulatory T cells in the liver-draining celiac lymph node following oral antigen administration. *Immunology* 116:362-372.
146. Iwata, M. 2009. Retinoic acid production by intestinal dendritic cells and its role in T-cell trafficking. *Semin Immunol* 21:8-13.
147. Miyamoto, K., C. I. Kingsley, X. Zhang, C. Jabs, L. Izikson, R. A. Sobel, H. L. Weiner, V. K. Kuchroo, and A. H. Sharpe. 2005. The ICOS molecule plays a crucial role in the development of mucosal tolerance. *J Immunol* 175:7341-7347.
148. Nilsen, E. M., F. E. Johansen, F. L. Jahnsen, K. E. Lundin, T. Scholz, P. Brandtzaeg, and G. Haraldsen. 1998. Cytokine profiles of cultured microvascular endothelial cells from the human intestine. *Gut* 42:635-642.
149. Sun, C. M., J. A. Hall, R. B. Blank, N. Bouladoux, M. Oukka, J. R. Mora, and Y. Belkaid. 2007. Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. *J Exp Med* 204:1775-1785.
150. Coombes, J. L., K. R. Siddiqui, C. V. Arancibia-Carcamo, J. Hall, C. M. Sun, Y. Belkaid, and F. Powrie. 2007. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. *J Exp Med* 204:1757-1764.
151. Robinson, P. W., S. J. Green, C. Carter, J. Coadwell, and P. J. Kilshaw. 2001. Studies on transcriptional regulation of the mucosal T-cell integrin alphaEbeta7 (CD103). *Immunology* 103:146-154.
152. Toby. 2002. Manipulation of dendritic cells as an approach to improved outcomes in transplantation. *Expert Review in Molecular Medicine* 1462-3994.
153. Pasare, C., and R. Medzhitov. 2003. Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells. *Science* 299:1033-1036.
154. Apostolou, I., and H. von Boehmer. 2004. In vivo instruction of suppressor commitment in naive T cells. *J Exp Med* 199:1401-1408.
155. Kretschmer, K., I. Apostolou, D. Hawiger, K. Khazaie, M. C. Nussenzweig, and H. von Boehmer. 2005. Inducing and expanding regulatory T cell populations by foreign antigen. *Nat Immunol* 6:1219-1227.
156. Kretschmer, K., I. Apostolou, E. Jaeckel, K. Khazaie, and H. von Boehmer. 2006. Making regulatory T cells with defined antigen specificity: role in autoimmunity and cancer. *Immunol Rev* 212:163-169.
157. Picca, C. C., J. Larkin, 3rd, A. Boesteanu, M. A. Lerman, A. L. Rankin, and A. J. Caton. 2006. Role of TCR specificity in CD4+ CD25+ regulatory T-cell selection. *Immunol Rev* 212:74-85.
158. Belkaid, Y. 2007. Regulatory T cells and infection: a dangerous necessity. *Nat Rev Immunol* 7:875-888.
159. Grainger, J. R., J. A. Hall, N. Bouladoux, G. Oldenhove, and Y. Belkaid. 2010. Microbe-dendritic cell dialog controls regulatory T-cell fate. *Immunol Rev* 234:305-316.
160. Mills, K. H., and P. McGuirk. 2004. Antigen-specific regulatory T cells--their induction and role in infection. *Semin Immunol* 16:107-117.
161. D'Ambrosio, A., M. Colucci, O. Pugliese, F. Quintieri, and M. Boirivant. 2008. Cholera toxin B subunit promotes the induction of regulatory T cells by preventing human dendritic cell maturation. *J Leukoc Biol* 84:661-668.

162. Fleming, S. B., D. M. Haig, P. Nettleton, H. W. Reid, C. A. McCaughan, L. M. Wise, and A. Mercer. 2000. Sequence and functional analysis of a homolog of interleukin-10 encoded by the parapoxvirus orf virus. *Virus Genes* 21:85-95.
163. Gomez-Escobar, N., W. F. Gregory, and R. M. Maizels. 2000. Identification of tgh-2, a filarial nematode homolog of Caenorhabditis elegans daf-7 and human transforming growth factor beta, expressed in microfilarial and adult stages of Brugia malayi. *Infect Immun* 68:6402-6410.
164. Lavelle, E. C., E. McNeela, M. E. Armstrong, O. Leavy, S. C. Higgins, and K. H. Mills. 2003. Cholera toxin promotes the induction of regulatory T cells specific for bystander antigens by modulating dendritic cell activation. *J Immunol* 171:2384-2392.
165. McGuirk, P., C. McCann, and K. H. Mills. 2002. Pathogen-specific T regulatory 1 cells induced in the respiratory tract by a bacterial molecule that stimulates interleukin 10 production by dendritic cells: a novel strategy for evasion of protective T helper type 1 responses by Bordetella pertussis. *J Exp Med* 195:221-231.
166. Parsonnet, J., G. D. Friedman, D. P. Vandersteen, Y. Chang, J. H. Vogelman, N. Orentreich, and R. K. Sibley. 1991. Helicobacter pylori infection and the risk of gastric carcinoma. *N Engl J Med* 325:1127-1131.
167. Pritchard, D. M., and J. E. Crabtree. 2006. Helicobacter pylori and gastric cancer. *Curr Opin Gastroenterol* 22:620-625.
168. Li, W., M. Minohara, J. J. Su, T. Matsuoka, M. Osoegawa, T. Ishizu, and J. Kira. 2007. Helicobacter pylori infection is a potential protective factor against conventional multiple sclerosis in the Japanese population. *J Neuroimmunol* 184:227-231.
169. Gewirtz, A. T., Y. Yu, U. S. Krishna, D. A. Israel, S. L. Lyons, and R. M. Peek, Jr. 2004. Helicobacter pylori flagellin evades toll-like receptor 5-mediated innate immunity. *J Infect Dis* 189:1914-1920.
170. Moran, A. P., B. Lindner, and E. J. Walsh. 1997. Structural characterization of the lipid A component of Helicobacter pylori rough- and smooth-form lipopolysaccharides. *J Bacteriol* 179:6453-6463.
171. Kim, J. M., J. S. Kim, D. Y. Yoo, S. H. Ko, N. Kim, H. Kim, and Y. J. Kim. 2011. Stimulation of dendritic cells with Helicobacter pylori vacuolating cytotoxin negatively regulates their maturation via the restoration of E2F1. *Clin Exp Immunol* 166:34-45.
172. Tisch, R. Immunogenic versus tolerogenic dendritic cells: a matter of maturation. *Int Rev Immunol* 29:111-118.
173. Marie, J. C., J. J. Letterio, M. Gavin, and A. Y. Rudensky. 2005. TGF-beta1 maintains suppressor function and Foxp3 expression in CD4+CD25+ regulatory T cells. *J Exp Med* 201:1061-1067.
174. Yamagiwa, S., J. D. Gray, S. Hashimoto, and D. A. Horwitz. 2001. A role for TGF-beta in the generation and expansion of CD4+CD25+ regulatory T cells from human peripheral blood. *J Immunol* 166:7282-7289.
175. Beswick, E. J., I. V. Pinchuk, R. B. Earley, D. A. Schmitt, and V. E. Reyes. 2011. Role of gastric epithelial cell-derived transforming growth factor beta in reduced CD4+ T cell proliferation and development of regulatory T cells during Helicobacter pylori infection. *Infect Immun* 79:2737-2745.
176. Bimczok, D., J. M. Grams, R. D. Stahl, K. B. Waites, L. E. Smythies, and P. D. Smith. 2011. Stromal regulation of human gastric dendritic cells restricts the Th1 response to Helicobacter pylori. *Gastroenterology* 141:929-938.

177. Lindholm, C., M. Quiding-Jarbrink, H. Lonroth, A. Hamlet, and A. M. Svennerholm. 1998. Local cytokine response in Helicobacter pylori-infected subjects. *Infect Immun* 66:5964-5971.
178. Lindholm, C., M. Quiding-Jarbrink, H. Lonroth, and A. M. Svennerholm. 2001. Induction of chemokine and cytokine responses by Helicobacter pylori in human stomach explants. *Scand J Gastroenterol* 36:1022-1029.
179. Maldonado, R. A., and U. H. von Andrian. 2010. How tolerogenic dendritic cells induce regulatory T cells. *Adv Immunol* 108:111-165.
180. Shevach, E. M. 2009. Mechanisms of foxp3+ T regulatory cell-mediated suppression. *Immunity* 30:636-645.
181. Kiriya, K., N. Watanabe, A. Nishio, K. Okazaki, M. Kido, K. Saga, J. Tanaka, T. Akamatsu, S. Ohashi, M. Asada, T. Fukui, and T. Chiba. 2007. Essential role of Peyer's patches in the development of Helicobacter-induced gastritis. *Int Immunol* 19:435-446.
182. Nagai, S., H. Mimuro, T. Yamada, Y. Baba, K. Moro, T. Nochi, H. Kiyono, T. Suzuki, C. Sasakawa, and S. Koyasu. 2007. Role of Peyer's patches in the induction of Helicobacter pylori-induced gastritis. *Proc Natl Acad Sci U S A* 104:8971-8976.
183. Toller, I. M., M. Altmeyer, E. Kohler, M. O. Hottiger, and A. Muller. 2011. Inhibition of ADP ribosylation prevents and cures helicobacter-induced gastric preneoplasia. *Cancer Res* 70:5912-5922.
184. Toller, I. M., I. Hitzler, A. Sayi, and A. Mueller. 2011. Prostaglandin E2 prevents Helicobacter-induced gastric preneoplasia and facilitates persistent infection in a mouse model. *Gastroenterology* 138:1455-1467, 1467 e1451-1454.
185. Sayi, A., E. Kohler, I. Hitzler, I. Arnold, R. Schwendener, H. Rehrauer, and A. Muller. 2009. The CD4+ T cell-mediated IFN-gamma response to Helicobacter infection is essential for clearance and determines gastric cancer risk. *J Immunol* 182:7085-7101.
186. Lang, R., D. Patel, J. J. Morris, R. L. Rutschman, and P. J. Murray. 2002. Shaping gene expression in activated and resting primary macrophages by IL-10. *J Immunol* 169:2253-2263.
187. Moore, K. W., R. de Waal Malefyt, R. L. Coffman, and A. O'Garra. 2001. Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol* 19:683-765.
188. Fainaru, O., T. Shay, S. Hantisteanu, D. Goldenberg, E. Domany, and Y. Groner. 2007. TGFbeta-dependent gene expression profile during maturation of dendritic cells. *Genes Immun* 8:239-244.
189. Karlsson, G., Y. Liu, J. Larsson, M. J. Goumans, J. S. Lee, S. S. Thorgeirsson, M. Ringner, and S. Karlsson. 2005. Gene expression profiling demonstrates that TGF-beta1 signals exclusively through receptor complexes involving Alk5 and identifies targets of TGF-beta signaling. *Physiol Genomics* 21:396-403.
190. Sargent, J. L., A. Milano, S. Bhattacharyya, J. Varga, M. K. Connolly, H. Y. Chang, and M. L. Whitfield. A TGFbeta-responsive gene signature is associated with a subset of diffuse scleroderma with increased disease severity. *J Invest Dermatol* 130:694-705.
191. Ismail, H. F., P. Fick, J. Zhang, R. G. Lynch, and D. J. Berg. 2003. Depletion of neutrophils in IL-10(-/-) mice delays clearance of gastric Helicobacter infection and decreases the Th1 immune response to Helicobacter. *J Immunol* 170:3782-3789.
192. Cheng, H. H., C. S. Chang, H. J. Wang, and W. C. Wang. 2011. Interleukin-1beta and -10 polymorphisms influence erosive reflux esophagitis and gastritis in Taiwanese patients. *J Gastroenterol Hepatol* 25:1443-1451.

193. Gao, L., M. N. Weck, A. Nieters, and H. Brenner. 2009. Association between a pro-inflammatory genetic profile and the risk of chronic atrophic gastritis among older adults from Germany. *Eur J Cancer* 45:428-434.
194. Sturlan, S., G. Oberhuber, B. G. Beinhauer, B. Tichy, S. Kappel, J. Wang, and M. A. Rogy. 2001. Interleukin-10-deficient mice and inflammatory bowel disease associated cancer development. *Carcinogenesis* 22:665-671.
195. Sugimoto, M., T. Furuta, N. Shirai, A. Nakamura, M. Kajimura, H. Sugimura, and A. Hishida. 2007. Effects of interleukin-10 gene polymorphism on the development of gastric cancer and peptic ulcer in Japanese subjects. *J Gastroenterol Hepatol* 22:1443-1449.
196. Serrano, C. A., E. Talesnik, A. Pena, A. Rollan, I. Duarte, J. Torres, D. Majerson, H. Einisman, P. Viviani, and P. R. Harris. 2011. Inverse correlation between allergy markers and Helicobacter pylori infection in children is associated with elevated levels of TGF-beta. *Eur J Gastroenterol Hepatol* 23:656-663.
197. Wu, M. S., J. T. Lin, P. N. Hsu, C. Y. Lin, Y. T. Hsieh, Y. H. Chiu, P. R. Hsueh, and K. W. Liao. 2007. Preferential induction of transforming growth factor-beta production in gastric epithelial cells and monocytes by Helicobacter pylori soluble proteins. *J Infect Dis* 196:1386-1393.
198. Veldhoen, M., R. J. Hocking, C. J. Atkins, R. M. Locksley, and B. Stockinger. 2006. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity* 24:179-189.
199. Xu, L., A. Kitani, I. Fuss, and W. Strober. 2007. Cutting edge: regulatory T cells induce CD4+CD25-Foxp3- T cells or are self-induced to become Th17 cells in the absence of exogenous TGF-beta. *J Immunol* 178:6725-6729.
200. Yang, X. O., R. Nurieva, G. J. Martinez, H. S. Kang, Y. Chung, B. P. Pappu, B. Shah, S. H. Chang, K. S. Schluns, S. S. Watowich, X. H. Feng, A. M. Jetten, and C. Dong. 2008. Molecular antagonism and plasticity of regulatory and inflammatory T cell programs. *Immunity* 29:44-56.
201. Harrington, L. E., R. D. Hatton, P. R. Mangan, H. Turner, T. L. Murphy, K. M. Murphy, and C. T. Weaver. 2005. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol* 6:1123-1132.
202. Park, H., Z. Li, X. O. Yang, S. H. Chang, R. Nurieva, Y. H. Wang, Y. Wang, L. Hood, Z. Zhu, Q. Tian, and C. Dong. 2005. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol* 6:1133-1141.
203. Deknuydt, F., G. Bioley, D. Valmori, and M. Ayyoub. 2009. IL-1beta and IL-2 convert human Treg into T(H)17 cells. *Clin Immunol* 131:298-307.
204. Li, L., J. Kim, and V. A. Boussiotis. 2010. IL-1beta-mediated signals preferentially drive conversion of regulatory T cells but not conventional T cells into IL-17-producing cells. *J Immunol* 185:4148-4153.
205. Shaw, M. H., N. Kamada, Y. G. Kim, and G. Nunez. 2012. Microbiota-induced IL-1beta, but not IL-6, is critical for the development of steady-state TH17 cells in the intestine. *J Exp Med* 209:251-258.
206. Franchi, L., T. Eigenbrod, R. Munoz-Planillo, and G. Nunez. 2009. The inflammasome: a caspase-1-activation platform that regulates immune responses and disease pathogenesis. *Nat Immunol* 10:241-247.
207. Amer, A., L. Franchi, T. D. Kanneganti, M. Body-Malapel, N. Ozoren, G. Brady, S. Meshinchi, R. Jagirdar, A. Gewirtz, S. Akira, and G. Nunez. 2006. Regulation of Legionella phagosome maturation and infection through flagellin and host Ipaf. *J Biol Chem* 281:35217-35223.

208. Broz, P., and D. M. Monack. 2011. Molecular mechanisms of inflammasome activation during microbial infections. *Immunol Rev* 243:174-190.
209. Muller, A. J., C. Hoffmann, M. Galle, A. Van Den Broeke, M. Heikenwalder, L. Falter, B. Misselwitz, M. Kremer, R. Beyaert, and W. D. Hardt. 2009. The S. Typhimurium effector SopE induces caspase-1 activation in stromal cells to initiate gut inflammation. *Cell Host Microbe* 6:125-136.
210. Sutterwala, F. S., L. A. Mijares, L. Li, Y. Ogura, B. I. Kazmierczak, and R. A. Flavell. 2007. Immune recognition of Pseudomonas aeruginosa mediated by the IPAF/NLRC4 inflammasome. *J Exp Med* 204:3235-3245.
211. Hitzler, I., A. Sayi, E. Kohler, D. B. Engler, K. N. Koch, W. D. Hardt, and A. Muller. 2012. Caspase-1 Has Both Proinflammatory and Regulatory Properties in Helicobacter Infections, Which Are Differentially Mediated by Its Substrates IL-1beta and IL-18. *J Immunol* 188:3594-3602.
212. El-Omar, E. M., M. Carrington, W. H. Chow, K. E. McColl, J. H. Bream, H. A. Young, J. Herrera, J. Lissowska, C. C. Yuan, N. Rothman, G. Lanyon, M. Martin, J. F. Fraumeni, Jr., and C. S. Rabkin. 2001. The role of interleukin-1 polymorphisms in the pathogenesis of gastric cancer. *Nature* 412:99.
213. Nishimura, M., H. Kawakami, O. Komure, H. Maruyama, H. Morino, Y. Izumi, S. Nakamura, R. Kaji, and S. Kuno. 2002. Contribution of the interleukin-1beta gene polymorphism in multiple system atrophy. *Mov Disord* 17:808-811.
214. Santtila, S., K. Savinainen, and M. Hurme. 1998. Presence of the IL-1RA allele 2 (IL1RN*2) is associated with enhanced IL-1beta production in vitro. *Scand J Immunol* 47:195-198.
215. Arnold, H., Müller. 2012. The immunomodulatory properties of *Helicobacter pylori* confer protection against allergic and chronic inflammatory disorders. *Cellular and Infection Microbiology* 2/10:1-9.
216. Salama, N. R., G. Otto, L. Tompkins, and S. Falkow. 2001. Vacuolating cytotoxin of Helicobacter pylori plays a role during colonization in a mouse model of infection. *Infect Immun* 69:730-736.
217. Covacci, A., J. L. Telford, G. Del Giudice, J. Parsonnet, and R. Rappuoli. 1999. Helicobacter pylori virulence and genetic geography. *Science* 284:1328-1333.
218. Galmiche, A., J. Rassow, A. Doye, S. Cagnol, J. C. Chambard, S. Contamin, V. de Thillot, I. Just, V. Ricci, E. Solcia, E. Van Obberghen, and P. Boquet. 2000. The N-terminal 34 kDa fragment of Helicobacter pylori vacuolating cytotoxin targets mitochondria and induces cytochrome c release. *EMBO J* 19:6361-6370.
219. Genisset, C., A. Puhar, F. Calore, M. de Bernard, P. Dell'Antone, and C. Montecucco. 2007. The concerted action of the Helicobacter pylori cytotoxin VacA and of the v-ATPase proton pump induces swelling of isolated endosomes. *Cell Microbiol* 9:1481-1490.
220. Gebert, B., W. Fischer, E. Weiss, R. Hoffmann, and R. Haas. 2003. Helicobacter pylori vacuolating cytotoxin inhibits T lymphocyte activation. *Science* 301:1099-1102.
221. Gerhard, M., C. Schmees, P. Voland, N. Endres, M. Sander, W. Reindl, R. Rad, M. Oelsner, T. Decker, M. Mempel, L. Hengst, and C. Prinz. 2005. A secreted low-molecular-weight protein from Helicobacter pylori induces cell-cycle arrest of T cells. *Gastroenterology* 128:1327-1339.
222. Schmees, C., C. Prinz, T. Treptau, R. Rad, L. Hengst, P. Voland, S. Bauer, L. Brenner, R. M. Schmid, and M. Gerhard. 2007. Inhibition of T-cell proliferation by Helicobacter pylori gamma-glutamyl transpeptidase. *Gastroenterology* 132:1820-1833.

223. Chevalier, C., J. M. Thiberge, R. L. Ferrero, and A. Labigne. 1999. Essential role of Helicobacter pylori gamma-glutamyltranspeptidase for the colonization of the gastric mucosa of mice. *Mol Microbiol* 31:1359-1372.
224. Raju, D., S. Hussey, M. Ang, M. R. Terebiznik, M. Sibony, E. Galindo-Mata, V. Gupta, S. R. Blanke, A. Delgado, J. Romero-Gallo, M. S. Ramjeet, H. Mascarenhas, R. M. Peek, P. Correa, C. Streutker, G. Hold, E. Kunstmann, T. Yoshimori, M. S. Silverberg, S. E. Girardin, D. J. Philpott, E. El Omar, and N. L. Jones. 2012. Vacuolating cytotoxin and variants in Atg16L1 that disrupt autophagy promote Helicobacter pylori infection in humans. *Gastroenterology* 142:1160-1171.
225. Zheng, P. Y., and N. L. Jones. 2003. Helicobacter pylori strains expressing the vacuolating cytotoxin interrupt phagosome maturation in macrophages by recruiting and retaining TACO (coronin 1) protein. *Cell Microbiol* 5:25-40.
226. Blaser, M. J., and S. Falkow. 2009. What are the consequences of the disappearing human microbiota? *Nat Rev Microbiol* 7:887-894.
227. Bach, J. F. 2002. The effect of infections on susceptibility to autoimmune and allergic diseases. *N Engl J Med* 347:911-920.
228. Linz, B., F. Balloux, Y. Moodley, A. Manica, H. Liu, P. Roumagnac, D. Falush, C. Stamer, F. Prugnolle, S. W. van der Merwe, Y. Yamaoka, D. Y. Graham, E. Perez-Trallero, T. Wadstrom, S. Suerbaum, and M. Achtman. 2007. An African origin for the intimate association between humans and Helicobacter pylori. *Nature* 445:915-918.

6. ACKNOWLEDGMENTS

First and foremost, I would like to thank Anne Müller for her tremendous scientific guidance, her addictive passion for science and her endless drive. Besides being an outstanding scientific supervisor, there were many occasions, when she supported me in questions of daily life. I really enjoyed working with Anne and will, at least some times, miss working in her team.

I would also like to acknowledge my Ph.D. committee members for their helpful suggestions and stimulating discussions during our meetings. Additionally, a lot of the performed experiments would have been postponed without the gender sharing of different mouse strains and reagents, especially by Prof. Dr. Burkhard Becher and PD. Dr. Lubor Borsig.

I am really grateful to my recent and former lab-mates, which all contributed to create an enjoyable atmosphere inside and outside the lab. Special thank goes to Isabelle Arnold and Esther Kohler who helped me tremendously getting started with my Ph.D. project.

I am grateful to all members of the Institute of Molecular Cancer Research, especially to Josef Jiricny for providing state of the art conditions for scientific research, and the Life Science Zurich Graduate School/Cancer PhD Program.

I would like to thank Heidi, Fritz and Sabina for being wonderful, understanding parents and sister. Without their financial support I would not have been able to become the first academic in our family (hell yeah..!). Further I would like to acknowledge my friends Bettina, Rudy, Dafi&Sahra&Lili, Marco&Scouler dudes, Claudio&Laura&Rocco, to let me off the hook whenever I had to postpone appointments due to lab work.

Last but not least, I would like to acknowledge Prof. Dr. Eric G. Berger and Prof. Dr. Jack Rohrer. Both of them are responsible for my belief and motivation to do a Ph.D.

7. APPENDIX

7.1 *HELICOBACTER PYLORI* γ-GLUTAMYL TRANSPEPTIDASE AND VACUOLATING CYTOTOXIN PROMOTE GASTRIC COLONIZATION THROUGH TOLEROGENIC RE-PROGRAMING OF DENDRITIC CELLS

Manuscript submitted to Proceedings of the National Academy of Sciences

Authors: Mathias Oertli, Manuel Noben, Daniela B. Engler, Raffaela Semper, Markus Gerhard, Sebastian Reuter, Joachim Maxeiner, Christian Taube, and Anne Müller

Contributions: I and MN designed, conducted and analyzed all experiments except for figure 7 (DBE, SR, JM, CT), RS and MG provided a GGT deficient mutant, AM suggested and planned experiments, helped with data analysis and wrote the manuscript

Helicobacter pylori γ-glutamyl transpeptidase and vacuolating cytotoxin promote gastric persistence and confer protection against asthma

Mathias Oertli[a], Manuel Noben[a], Daniela B. Engler[a], Raphaela P. Semper[b], Markus Gerhard[b], Sebastian Reuter[c], Joachim Maxeiner[c], Christian Taube[d], and Anne Müller[a]

[a]Institute of Molecular Cancer Research, University of Zurich, Zurich, Switzerland; [b]Institute of Microbiology, Immunology and Hygiene, Technical University of Munich, Germany, [c]Third Medical Clinic, University of Mainz, Germany, and [d]Department of Pulmonology, Leiden University Medical Center, The Netherlands

Correspondence: Anne Müller, Institute of Molecular Cancer Research, University of Zürich, Winterthurerstr. 190, 8057 Zürich, Switzerland, phone: +41 44 635 3474, email: mueller@imcr.uzh.ch

Running title: *H. pylori* GGT and VacA promote immune tolerance

Classification: Biological Sciences, Microbiology

Abstract word count: 241; total character count: 38.814 including spaces

Abstract

Infection with the gastric bacterial pathogen *Helicobacter pylori* is typically contracted in early childhood and often persists for decades. The immunomodulatory properties of *H. pylori* that allow it to colonize humans persistently are further believed to account for *H. pylori*'s protective effects against allergic and chronic inflammatory diseases. *H. pylori* infection efficiently re-programs dendritic cells (DCs) towards a tolerogenic phenotype and induces regulatory T-cells with highly suppressive activity in models of allergen-induced asthma. We show here that two *H. pylori* virulence determinants, the γ-glutamyl transpeptidase GGT and the vacuolating cytotoxin VacA, contribute critically and non-redundantly to *H. pylori*'s tolerizing effects on DCs *in vitro* and *in vivo*. The tolerance-promoting effects of both factors are independent of their described suppressive activity on T-cells. Isogenic mutants lacking GGT or VacA are incapable of preventing LPS-induced DC maturation, and fail to drive DC tolerization as assessed by induction of Treg properties in co-cultured naive T-cells. The Δ*ggt* and Δ*vacA* mutants colonize mice at significantly reduced levels, induce stronger Th1 and Th17 responses and/or trigger more severe gastric pathology. Both factors are required for the efficient induction of Tregs with suppressive activity in allergen-induced asthma. The defects of the Δ*ggt* mutant *in vitro* and *in vivo* are phenocopied by pharmacological inhibition of the transpeptidase activity of GGT in all read outs. In conclusion, our results reveal the molecular players and mechanistic basis for *H. pylori*-induced immunomodulation, promoting persistent infection and conferring protection against allergic asthma.

Introduction

Helicobacter pylori is a persistent bacterial pathogen colonizing the gastric mucosa of humans. It is typically acquired in early childhood (1) and, in the absence of antibiotic therapy, may persist for the entire life span of the host (2, 3). The extraordinary ability of *H. pylori* to resist a vigorous adaptive immune response driven in large part by Th1 and/or Th17-polarized effector T-cells (4-7) has been attributed to its perfect adaptation to -and manipulation of- the human innate and adaptive immune systems (8, 9). *H. pylori* has colonized its human host for at least 60.000 years (10) and during this long period of co-evolution has evolved elaborate ways to systemically manipulate adaptive immune responses and to promote its persistence through the preferential induction of regulatory T-cell (Treg) over T-effector cell responses. Treg-predominant responses are characteristic of heavily colonized, but asymptomatic carriers (4) and of children with particularly mild forms of *Helicobacter*-associated gastritis (11). Several recent functional studies using experimentally infected animals have implicated Tregs and dendritic cells (DCs) with "tolerogenic" activity in mediating the local and systemic immunomodulatory effects of *H. pylori* infection (12-15). The depletion of Tregs in a genetic model resulted in spontaneous clearance of the infection (12) and greatly improved the efficacy of an *H. pylori* vaccine (13). *H. pylori*-induced Tregs differentiate in the periphery as a result of their priming by tolerogenic DCs (16), which convert naive T-cells into FoxP3$^+$ Tregs through antigen presentation in the absence of co-stimulatory signals or cytokines (16, 17). We have shown recently that *H. pylori* exposure re-programs DCs towards a tolerance-promoting phenotype *in vitro* and *in vivo*; *H. pylori*-experienced DCs fail to induce T-cell effector functions, but rather acquire the ability to induce FoxP3 and CD25 expression in co-cultured naive T-cells (15). Consistent with a critical role for DCs in the development of *H. pylori*-specific immune tolerance, the systemic depletion of DCs breaks tolerance and facilitates clearance of the bacteria (15).

Here, we describe for the first time the role of two *H. pylori* virulence determinants, VacA and GGT, in DC re-programming and in the development of immune tolerance *in vitro* and *in vivo*.

We show that both factors independently interfere with DC maturation and thereby contribute to DC tolerization. Specific deletion of the *ggt* and *vacA* genes or the pharmacological inhibition of GGT activity impair the ability of *H. pylori* to tolerize DCs *in vitro* and *in vivo* and to generate Tregs with suppressive activity. The isogenic Δ*ggt* and Δ*vacA* mutants fail to colonize mice persistently, and have lost the ability to protect against allergen-induced asthma. In conclusion, we have identified here a novel immunomodulatory mechanism of *H. pylori* that involves specific targeting and tolerogenic re-programming of DCs and possibly explains the extraordinary ability of these bacteria to persist in their mammalian host and, at the same time, to confer protection against allergen-specific T-cell responses and asthma.

Results

The tolerogenic re-programming of DCs by *H. pylori* depends on the virulence determinants γ-glutamyl transpeptidase and vacuolating cytotoxin

To assess the contribution of specific *H. pylori* virulence determinants to DC tolerization, we compared various isogenic mutants to the respective parental wild type strains with respect to their ability to (a) induce DC maturation and to (b) actively suppress LPS-induced DC maturation. Mutants lacking components of the type IV secretion system, or the type IV-secreted effector CagA, or one of several bacterial adhesins did not differ from the corresponding wild type strains in these respects. In contrast, isogenic mutants lacking either of two secreted virulence factors, the γ-glutamyl-transpeptidase (GGT) or the vacuolating cytotoxin VacA, induced DC maturation more efficiently than wild type bacteria and failed to inhibit DC maturation induced by LPS as assessed by CD80 expression (Figure 1A) and IL-12 ELISA (Figure 1B); similar results were obtained in three different strain backgrounds (Figure 1; suppl. Figure 1). The combined deletion of *ggt* and *vacA* did not produce additive or synergistic effects (Figure 1A,B).

To obtain definitive proof that the unique semi-mature status of *H. pylori*-experienced DCs is required for their tolerance-promoting activity, we measured FoxP3 and CD25 expression in co-cultured naive T-cells. Bone marrow-derived DCs that had been exposed to *H. pylori* efficiently induced T-cellular FoxP3/CD25 expression, which was abrogated by the forced (partial) maturation of *H. pylori*-infected DCs by simultaneous treatment with increasing doses of *E. coli* (suppl. Figure 2A,B). To assess the effects of LPS treatment *in vivo*, C57BL/6 mice were infected with *H. pylori* during the neonatal period, i.e. at a time when *H. pylori* exposure is known to induce immune tolerance (12), and then subjected to twice-weekly sublethal intraperitoneal doses of *E. coli* LPS. The LPS treatment induced DC maturation in the gut-draining mesenteric lymph nodes, the sites of *H. pylori*-specific T-cell priming (suppl. Figure 2C). LPS-treated mice controlled the infection more effectively, and exhibited higher gastric mucosal leukocyte and $CD4^+$ T-cell infiltration than untreated infected controls; more Th1 and Th17 infiltration and higher inflammation scores was seen as well (suppl. Figure 2D-I). In summary, the results suggest that the forced maturation of DCs with sublethal LPS breaks *H. pylori*-specific, neonatally acquired immune tolerance.

To examine the functional contribution of VacA and GGT to *H. pylori*-induced DC tolerogenicity *in vitro*, bone marrow-derived DCs were infected o/n with either Δ*ggt* or Δ*vacA* mutant bacteria or the corresponding parental wild type strain and then co-cultured with naive T-cells. DCs that had been exposed to the mutant bacteria were much less capable of inducing T-cellular CD25 and FoxP3 expression than wild type-infected DCs; this was true in all three strain backgrounds analyzed (Figure 1C,D, suppl. Figure 3A,B). Again, a double mutant lacking both GGT and VacA did not exhibit a stronger phenotype than the single mutants (suppl. Figure 3A). To clarify whether the catalytic activity of GGT is required for GGT's tolerogenic effects on DCs, we infected DCs with wild type *H. pylori* in the presence or absence of the GGT inhibitor acivicin. The tolerogenic activity of *H. pylori*-infected DCs was strongly reduced by exposure to the inhibitor (Figure 1C,E). The combined results obtained with the isogenic mutants and the GGT inhibitor thus suggest that VacA and GGT both contribute to DC tolerogenicity, the latter through its enzymatic transpeptidase activity.

Efficient gastric colonization by *H. pylori* and tolerogenic re-programming of DCs *in vivo* requires VacA and GGT

Both VacA and GGT have been shown earlier to contribute to the ability of *H. pylori* to colonize the gastric mucosa of experimentally infected mice (18, 19). We have used the isogenic mutants generated for this study in the PMSS1 background to revisit the earlier findings and to examine a possible role of VacA and GGT in colonization and in tolerogenic DC re-programming *in vivo*. Consistent with the earlier reports, both the Δ*ggt* and the Δ*vacA* mutant exhibited significant defects in colonizing mice relative to the parental PMSS1 strain, with average colonization levels reduced by ~1 order of magnitude compared to those of the wild type bacteria at one month post infection (Figure 2A). The mutants' lower colonization levels were accompanied by stronger Th1 and Th17 responses in the mesenteric lymph nodes of the mutant-infected relative to wild type-infected animals (suppl. Figure 4A-D), suggesting that both mutants elicited more pronounced pathogen-specific T-cell responses. To assess whether the three strains differentially affected DC tolerogenicity, DCs were immunomagnetically isolated from single cell mesenteric lymph node preparations of individual infected mice, and subjected to the same co-culture protocol with naive T-cells as outlined earlier. Whereas DCs from wild type-infected mice efficiently induced T-cellular FoxP3/CD25 expression, this was not observed with DCs from uninfected controls, or with DCs from mice infected with the Δ*ggt* and the Δ*vacA* mutants (Figure 2B, C). To confirm the phenotypes of both mutants in longer-term infections, and to assess possible differential effects on gastric histopathology, mice were infected with PMSS1 or the Δ*ggt* and the Δ*vacA* mutants for two months. At this later time point, seven of the ten Δ*ggt*-infected mice had cleared the infection, and the Δ*vacA*-infected animals still consistently had lower colonization levels than the wild type-infected controls (Figure 2D). Interestingly, whereas the wild type-infected animals exhibited mild to moderate inflammation and beginning preneoplastic changes at most, the Δ*vacA*-infected animals were characterized by severe inflammation and strongly aggravated pre-neoplastic pathology (Figure 2E,F). The Δ*ggt*-infected mice exhibited only mild residual inflammatory infiltrates (Figure 2E,F). In conclusion, both VacA

and GGT contribute critically to DC tolerization *in vitro* and *in vivo*, and this likely explains the stronger *H. pylori*-specific Th1/Th17 responses, the more efficient clearance of the mutant bacteria relative to their wild type counterparts, and the more pronounced gastric pathology observed with the Δ*vacA* mutant.

DC-mediated, neonatally acquired immune tolerance to *H. pylori* infection requires VacA and the enzymatic activity of GGT

The outcome of the *H. pylori*/ host interaction in experimentally infected mice is determined in large part by the age at the time of infection. Mice that are experimentally infected as neonates develop only mild gastritis and are completely protected from atrophy and preneoplasia (12). We have previously attributed this differential disease susceptibility to the development of Treg-mediated, peripheral immune tolerance in neonatally infected mice (12). To assess the contribution of GGT and VacA to neonatally acquired immune tolerance, we infected newborn mice with either wild type PMSS1, PMSS1Δ*ggt* or PMSS1Δ*vacA* and assessed their colonization levels at various time points post infection. Interestingly, *H. pylori* Δ*ggt* completely failed to colonize neonatal mice, i.e. no colonies were retrieved at one month post infection (suppl. Figure 5A). Mice infected with *H. pylori* Δ*vacA* were colonized, but at lower levels than their wild type-infected counterparts, at both one month and two months post infection (suppl. Figure 5A). To assess whether the enzymatic activity of GGT is required for the development and maintenance of neonatally acquired tolerance to the infection, we infected mice at one week of age, and either subjected them to regular intraperitoneal doses of the GGT inhibitor acivicin throughout the 6 week time course of infection, or to acivicin treatment during the last two weeks of the time course only. Of the five mice that received acivicin continuously, three were not colonized at all, and two exhibited minimal colonization; acivicin treatment during the last two weeks of infection led to a significant reduction in colonization, but did not induce clearance (suppl. Figure 5B). To assess whether acivicin treatment affected DC tolerogenicity, immunomagnetically isolated

DCs were subjected to co-culture with naive T-cells. Whereas DCs from neonatally infected mice that had not received acivicin efficiently induced T-cellular FoxP3 and CD25 expression, this was not observed with DCs from mice subjected to acivicin treatment, independent of the duration of the treatment (Figure 3A). In conclusion, GGT, via its enzymatic activity, is absolutely required for neonatal colonization, and targets DCs to promote neonatally acquired immune tolerance to *H. pylori* infection.

Both VacA and GGT have been implicated before in immunomodulation by *H. pylori* infection; the immunomodulatory activity of both factors has been attributed to suppressive effects on T-cells (20-22). To examine whether the tolerogenic effects of *H. pylori* on DCs *in vivo* are dependent on T-cells, we infected mice lacking α/β T-cells due to a targeted deletion of the TCR β-chain with wild type and mutant *H. pylori* PMSS1, as neonates and/or as adults. As noted before (12), the colonization efficiency of wild type bacteria does not differ between adult and neonatally infected TCR-$\beta^{-/-}$ mice (suppl. Figure 5C). *H. pylori* Δ*vacA* also colonizes these mice at high levels (suppl. Figure 5C); in contrast, the Δ*ggt* mutant failed to colonize any of the five mice analyzed. DCs from TCR-$\beta^{-/-}$ mice infected with wild type bacteria were significantly better inducers of FoxP3 and CD25 expression than DCs from uninfected TCR-$\beta^{-/-}$ donors (Figure 3B). Interestingly, DCs from neonatally infected TCR- $\beta^{-/-}$ donors were significantly more tolerogenic in the *ex vivo* assay than DCs from mice that had been infected as adults (Figure 3B), despite similar levels of colonization in both groups. Interestingly, DCs from Δ*vacA*-infected TCR- $\beta^{-/-}$ donors were incapable of inducing FoxP3 and CD25 expression in T-cells (Figure 3B), despite normal colonization. Similar results were obtained with DCs from infected OT-II mice that transgenically expressing a TCR specific for ovalbumin and therefore lack a normal diverse T-cell repertoire (suppl. Figure 6). The combined results indicate that the tolerization of DCs that is a hallmark of this infection is independent of T-cells, and suggest that VacA is critically involved in DC tolerization independent of its effects on T-cells.

VacA and GGT are essential for the generation of highly suppressive regulatory T-cells

One of the consequences of the efficient generation of suppressive Tregs during neonatal infection with *H. pylori* is the cross-protection against allergen-specific T-cell responses and asthma (23). To assess the suppressive activity of Tregs isolated from wild type and Δ*vacA*-infected donors in a mouse model of allergen-induced asthma, we performed adoptive transfer experiments (15, 23). C57BL/6 recipients were first sensitized and then challenged with ovalbumin. Mice subjected to this protocol were characterized by airway hyper-responsiveness to methacholine and broncho-alveolar immune cell infiltration, especially of eosinophils (compare negative controls, black circles, and positive controls, grey squares; Figure 4A-D), as well as histologically evident lung inflammation and goblet cell metaplasia (Figure 4E,F, suppl. Figure 7). The adoptive transfer of immunomagnetically isolated populations of mesenteric lymph node-derived $CD4^+CD25^+$ Tregs from neonatally infected donors to naive recipients before allergen challenge efficiently prevented the clinical and histopathological symptoms of allergic airway disease (Figure 4A-F, suppl. Figure 7). In contrast, Tregs from adult-infected donors or from donors infected during the neonatal period with *H. pylori* Δ*vacA* did not alleviate airway hyper-responsiveness and inflammation (Figure 4A-F, suppl. Figure 7). Recipients of Tregs from wild type neonatally infected donors in which IL-10 signaling was inactived by a blocking antibody did not benefit from the protective effects of the cells. We conclude from the combined results that neonatal infection with wild type, but not VacA-deficient bacteria yields highly suppressive Tregs, which on the one hand suppress clearance of the bacteria and promote persistent infection, and on the other hand efficiently suppress allergen-induced asthma through a mechanism involving IL-10 signaling.

Discussion

Several recent studies have independently documented the distinct ability of *H. pylori* to re-program DCs toward a tolerogenic phenotype *in vitro* and *in vivo*, ensuring persistence of the bacteria and cross-protecting against chronic inflammatory and autoimmune diseases (14, 15, 24, 25). *H. pylori*-experienced DCs appear to preferentially prime Treg over Th1 or Th17 responses and fail to produce pro-inflammatory cytokines (14, 15, 25). Consequently, the systemic depletion of DCs breaks *H. pylori*-specific tolerance, improves clearance of the infection in naive mice and enhances vaccine-induced protective immunity in immunized mice (13, 15). Here, we extend these findings and implicate two *H. pylori* virulence determinants, VacA and GGT, in DC tolerization. Both factors were previously known to facilitate murine colonization (18, 19), and to inhibit human T-cell activation (20-22, 26), although the two processes have to date not been causally linked *in vivo*. The evidence now provided here documents a novel role for VacA and GGT in DC tolerization and links the tolerizing effects of both factors on DCs to persistence: (a) the *H. pylori*-induced inhibition of DC maturation depends on the (non-redundant) activity of both factors, (b) the induction of Treg properties in naive T-cells by *H. pylori*-experienced, semi-mature DCs likewise depends on both factors, and strains lacking either VacA or GGT due to targeted gene deletion (c) fail to colonize mice at wild type levels, (d) induce stronger Th1 and Th17 responses and trigger more severe gastric pathology in the case of the Δ*vacA* mutant, and (e) fail to induce Tregs with suppressive activity in allergen-induced asthma. The defects of the Δ*ggt* mutant *in vitro* and *in vivo* are phenocopied by pharmacological inhibition of the transpeptidase activity of GGT in all read outs, suggesting that the enzymatic activity of GGT is required for its immunomodulatory effects. Interestingly, the tolerance-promoting effects of VacA- and GGT-proficient *H. pylori* on DCs were as pronounced in mouse strains with defective T-cell compartments as in wild type mice, ruling out a critical contribution of α/β T-cells to DC tolerization.

To our surprise, we found the phenotype of the Δ*ggt* mutant (and the effects of inhibitor treatment) to differ depending on the age of the mice at the time of infection. Whereas adult-infected animals support initial colonization, but have largely cleared the Δ*ggt* mutant by two months post infection, no mutant bacteria could ever be retrieved from neonatally infected mice, independent of the T-cell proficiency of the host. This observation argues that GGT has two mechanistically distinct roles in colonization and in persistence, enabling initial colonization of the specific environment of the juvenile stomach, and acting as an immunomodulator of the adult immune system. Both effects of GGT depend on its enzymatic activity. Exactly how GGT and VacA prevent DC maturation and promote DC tolerization remains to be clarified in detail. It is likely that both factors act on entirely different pathways. VacA has been described to prevent phagosome maturation in macrophages (27) and to prevent autophagy in epithelial cells (28), both of which could also be true in DCs and would explain their failure to respond properly to Δ*vacA H. pylori*. It will be interesting in this context to address whether the anion-selective membrane channel-forming activity of VacA residing in the amino-terminal p34 domain of VacA (29) is required for DC tolerization.

Despite its extraordinary host adaptation, *H. pylori* is lost from Western populations at an astonishing rate: in the United States, for example, *H. pylori* prevalence has declined from >50% at the beginning of the 20th century to <10% at its end (30). A series of epidemiological studies has documented an inverse epidemiological association between *H. pylori* infection and asthma and other allergic and chronic inflammatory disease manifestations, especially in children and young adults (31-35). Using an experimental model of allergic airway disease induced by ovalbumin-specific sensitization and challenge, we found that *H. pylori* infection protects mice against the clinical and histopathological symptoms of asthma (23). The same *H. pylori* factors facilitating persistent infection also appear to be required for protection against asthma. Indeed, it is conceivable that the immunomodulatory properties of *H. pylori* and of its persistence determinants can be harnessed to prevent or treat allergies and possibly other chronic inflammatory diseases that are of increasing public health importance in societies from which *H. pylori* is disappearing.

Materials and Methods

H. pylori strains and culture conditions

The following previously published or newly generated strains of *H. pylori* were used: PMSS1 (12), PMSS1Δ*vacA* (generated by natural transformation of G27Δ*vacA* gDNA into *H. pylori* strain SS1 and selection for kanamycin-resistant clones, followed by natural transformation of SS1Δ*vacA* gDNA into *H. pylori* strain PMSS1 and kanamycin selection), PMSS1Δ*ggt* (generated by natural transformation of G27Δ*ggt* gDNA (22)), G27 (36), G27Δ*vacA* (deficient for the vacuolating cytotoxin; generously provided by H. Kusters), P12, P12Δ*ggt*, P12Δ*vacA*, and P12Δ*ggt*/Δ*vacA* (obtained by replacing a P12Δ*vacA* mutant's *ggt* gene by a kanamycin resistance cassette and subsequent selection on chloramphenicol and kanamycin plates (all P12 strains described in (37)). *H. pylori* was grown on agar and in liquid culture as described (5).

Animal experimentation, lung and gastric histopathology, assessment of *H. pylori* colonization, flow cytometry, and DC/T-cell co-cultures

C57BL/6 wild-type, BL/6.TCR-$\beta^{-/-}$ and OT II TCR-transgenic mice were purchased from Charles River Laboratories (Sulzfeld, Germany). All mice were bred at a University of Zurich specific pathogen-free facility. Mixed gender groups were infected at either 7 days or 6 weeks of age with one orogastric dose of ~2×10^7 CFU *H. pylori* PMSS1 (12). *E. coli* LPS (Serotype 0111:B4, Sigma-Aldrich) was administered intraperitoneally every other day at 1 g/g body weight. Acivicin (Santa Cruz Biotechnology) was administered intraperitoneally every other day at 2 mg/g body weight. For asthma induction, mice were sensitized by two i.p. injections of 20 µg ovalbumin (Sigma-Aldrich) emulsified in 2.25 mg aluminum hydroxide (Alum Imject; Pierce) with a 2-week interval and challenged with 1% aerosolized ovalbumin using an ultrasonic nebulizer (NE-U17; Omron) for 20 min daily on days 14, 15 and 16 post (second) sensitization. IL-10R neutralizing antibody was administered twice (i.p., at 250

g/dose) on the first and third day of the challenge phase. For adoptive Treg transfers, $CD4^+CD25^+$ T-cells were immunomagnetically isolated (R&D Systems) from the pooled MLNs of five donors per group; 200.000 cells were intravenously injected two days before the first aerosol challenge. Airway resistance measurements were performed on anesthetized, intubated and mechanically ventilated mice (FlexiVent, Scireq, Montreal, Canada) in response to increasing doses of inhaled methacholine. Lungs were lavaged via the trachea with 1 ml PBS. BALF cells were counted using trypan blue dye exclusion. Differential cell counts of lymphocytes, neutrophils and eosinophils were performed on cytocentrifuged preparations stained with the Microscopy Hemacolor ®-Set (Merck). Lungs were fixed by inflation and immersion in 10% formalin and embedded in paraffin. Lung tissue sections were stained with H&E and periodic acid-Schiff and were examined in blinded fashion by two independent experimentors on a BX40 Olympus microscope. Peribronchial inflammation was scored on a scale from 0 to 4 on 5 randomly chosen areas per slide. PAS-positive goblet cells were quantified per 1 mm of basement membrane in the primary bronchus and several medium-sized bronchi using Soft Imaging Systems software (Stuttgart, Germany). Stomachs were retrieved and dissected longitudinally into equally sized pieces. For the quantitative assessment of *H. pylori* colonization, one stomach section was homogenized in Brucella broth and serial dilutions were plated on horse blood plates for colony counting as described (12). For the quantitative assessment of gastric histopathology, Giemsa-stained paraffin-embedded stomach sections were scored on a scale of 0–6 for the parameters of chronic inflammation, atrophy, epithelial hyperplasia, and metaplasia, as described in detail previously (5). All gastric histopathology images were taken at 100 or 200x final magnification on a Leica Leitz DM RB microscope equipped with a DFC 420C camera. Images were acquired using Leica Application Suite 3.3.0 software. All animal experimentation described here was reviewed and approved by the Zurich Cantonal veterinary office (63/2008 and 170/2009 to A.M.). Flow cytometry procedures and the preparation of BM-DCs, MLN-DCs and DC/T-cell co-cultures, as well as statistical analyses are described in the supplemental methods.

Acknowledgments

We are grateful to Karen Otteman and Katrin Koch for help with constructing the PMSS1Δ*vacA* mutant strain and to Esther Kohler for expert technical assistance.

References

1. Weyermann M, Rothenbacher D, Brenner H (2009) Acquisition of Helicobacter pylori infection in early childhood: independent contributions of infected mothers, fathers, and siblings. *Am J Gastroenterol* 104:182-189.
2. Morelli G, *et al.* (2010) Microevolution of Helicobacter pylori during prolonged infection of single hosts and within families. *PLoS Genet* 6:e1001036.
3. Suerbaum S,Josenhans C (2007) Helicobacter pylori evolution and phenotypic diversification in a changing host. *Nat Rev Microbiol* 5:441-452.
4. Robinson K, *et al.* (2008) Helicobacter pylori-induced peptic ulcer disease is associated with inadequate regulatory T cell responses. *Gut* 57:1375-1385.
5. Sayi A, *et al.* (2009) The CD4+ T cell-mediated IFN-gamma response to Helicobacter infection is essential for clearance and determines gastric cancer risk. *J Immunol* 182:7085-7101.
6. Algood HM, *et al.* (2009) Regulation of gastric B cell recruitment is dependent on IL-17 receptor A signaling in a model of chronic bacterial infection. *J Immunol* 183:5837-5846.
7. Shi Y, *et al.* (2010) Helicobacter pylori-induced Th17 responses modulate Th1 cell responses, benefit bacterial growth, and contribute to pathology in mice. *J Immunol* 184:5121-5129.
8. Muller A, Oertli M, Arnold IC (2011) H. pylori exploits and manipulates innate and adaptive immune cell signaling pathways to establish persistent infection. *Cell Commun Signal* 9:25.
9. Muller A,Solnick JV (Inflammation, immunity, and vaccine development for Helicobacter pylori. *Helicobacter* 16 Suppl 1:26-32.
10. Linz B, *et al.* (2007) An African origin for the intimate association between humans and Helicobacter pylori. *Nature* 445:915-918.
11. Harris PR, *et al.* (2008) Helicobacter pylori gastritis in children is associated with a regulatory T-cell response. *Gastroenterology* 134:491-499.
12. Arnold IC, *et al.* (2011) Tolerance rather than immunity protects from Helicobacter pylori-induced gastric preneoplasia. *Gastroenterology* 140:199-209.
13. Hitzler I, Oertli M, Becher B, Agger EM, Müller A (2011) Dendritic Cells Prevent Rather Than Promote Immunity Conferred by a Helicobacter Vaccine Using a Mycobacterial Adjuvant. *Gastroenterology* 141:186-196.
14. Kao JY, *et al.* (2010) Helicobacter pylori immune escape is mediated by dendritic cell-induced Treg skewing and Th17 suppression in mice. *Gastroenterology* 138:1046-1054.
15. Oertli M, *et al.* (2012) DC-derived IL-18 drives Treg differentiation, murine Helicobacter pylori-specific immune tolerance, and asthma protection. *J Clin Invest* 122:1082-1096.
16. Maldonado RA,von Andrian UH (2010) How tolerogenic dendritic cells induce regulatory T cells. *Adv Immunol* 108:111-165.
17. Kretschmer K, *et al.* (2005) Inducing and expanding regulatory T cell populations by foreign antigen. *Nat Immunol* 6:1219-1227.
18. Chevalier C, Thiberge JM, Ferrero RL, Labigne A (1999) Essential role of Helicobacter pylori gamma-glutamyltranspeptidase for the colonization of the gastric mucosa of mice. *Mol Microbiol* 31:1359-1372.
19. Salama NR, Otto G, Tompkins L, Falkow S (2001) Vacuolating cytotoxin of Helicobacter pylori plays a role during colonization in a mouse model of infection. *Infect Immun* 69:730-736.
20. Boncristiano M, *et al.* (2003) The Helicobacter pylori vacuolating toxin inhibits T cell activation by two independent mechanisms. *J Exp Med* 198:1887-1897.
21. Gebert B, Fischer W, Weiss E, Hoffmann R, Haas R (2003) Helicobacter pylori vacuolating cytotoxin inhibits T lymphocyte activation. *Science* 301:1099-1102.
22. Schmees C, *et al.* (2007) Inhibition of T-cell proliferation by Helicobacter pylori gamma-glutamyl transpeptidase. *Gastroenterology* 132:1820-1833.

23. Arnold IC, *et al.* (2011) Helicobacter pylori infection prevents allergic asthma in mouse models through the induction of regulatory T cells. *J Clin Invest* 121:3088-3093.
24. Luther J, *et al.* (2011) Helicobacter pylori DNA decreases pro-inflammatory cytokine production by dendritic cells and attenuates dextran sodium sulphate-induced colitis. *Gut* 60:1479-1486.
25. Wang YH, Gorvel JP, Chu YT, Wu JJ, Lei HY (2010) Helicobacter pylori impairs murine dendritic cell responses to infection. *PLoS One* 5:e10844.
26. Sundrud MS, Torres VJ, Unutmaz D, Cover TL (2004) Inhibition of primary human T cell proliferation by Helicobacter pylori vacuolating toxin (VacA) is independent of VacA effects on IL-2 secretion. *Proc Natl Acad Sci U S A* 101:7727-7732.
27. Zheng PY,Jones NL (2003) Helicobacter pylori strains expressing the vacuolating cytotoxin interrupt phagosome maturation in macrophages by recruiting and retaining TACO (coronin 1) protein. *Cell Microbiol* 5:25-40.
28. Raju D, *et al.* (Vacuolating cytotoxin and variants in Atg16L1 that disrupt autophagy promote Helicobacter pylori infection in humans. *Gastroenterology* 142:1160-1171.
29. McClain MS, *et al.* (2003) Essential role of a GXXXG motif for membrane channel formation by Helicobacter pylori vacuolating toxin. *J Biol Chem* 278:12101-12108.
30. Blaser MJ,Falkow S (2009) What are the consequences of the disappearing human microbiota? *Nat Rev Microbiol* 7:887-894.
31. Amberbir A, *et al.* (2011) Effects of Helicobacter pylori, geohelminth infection and selected commensal bacteria on the risk of allergic disease and sensitization in 3-year-old Ethiopian children. *Clin Exp Allergy*.
32. Chen Y,Blaser MJ (2007) Inverse associations of Helicobacter pylori with asthma and allergy. *Arch Intern Med* 167:821-827.
33. Chen Y,Blaser MJ (2008) Helicobacter pylori colonization is inversely associated with childhood asthma. *J Infect Dis* 198:553-560.
34. Luther J, Dave M, Higgins PD, Kao JY (2010) Association between Helicobacter pylori infection and inflammatory bowel disease: a meta-analysis and systematic review of the literature. *Inflamm Bowel Dis* 16:1077-1084.
35. Reibman J, *et al.* (2008) Asthma is inversely associated with Helicobacter pylori status in an urban population. *PLoS One* 3:e4060.
36. Censini S, *et al.* (1996) cag, a pathogenicity island of Helicobacter pylori, encodes type I-specific and disease-associated virulence factors. *Proc Natl Acad Sci U S A* 93:14648-14653.
37. Fassi Fehri L, *et al.* (2010) Helicobacter pylori induces miR-155 in T cells in a cAMP-Foxp3-dependent manner. *PLoS One* 5:e9500.

Figures

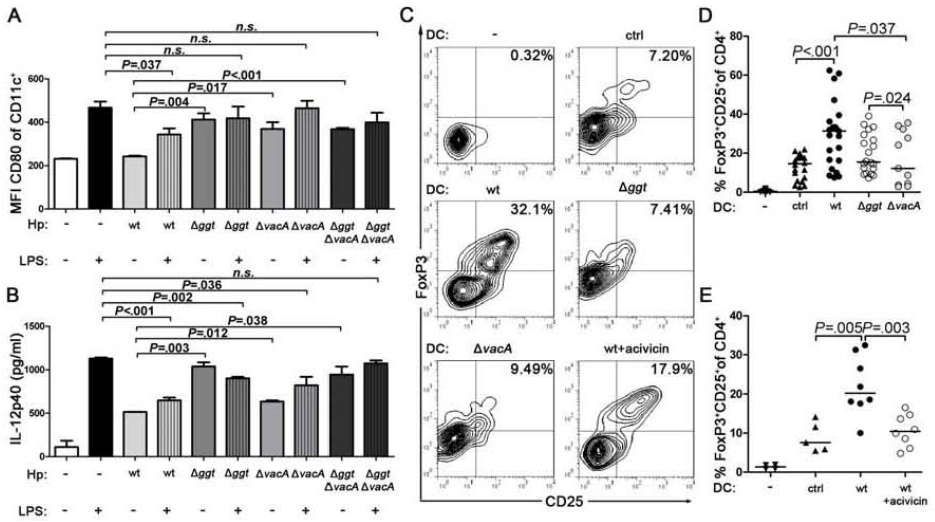


Figure 1. VacA and the enzymatic activity of GGT are required for DC tolerization. A,B, BM-DCs were infected with *H. pylori* P12, P12Δ*ggt*, P12Δ*vacA* or P12Δ*ggt*Δ*vacA* (MOI 50) and/or treated with 0.5 g/ml *E. coli* LPS for 16h prior to the flow cytometric analysis of CD11c and CD80 expression (A) and the analysis of IL-12p40 secretion by ELISA (B). MFI, mean fluorescence intensity. Data are representative of four independent experiments, and represented as means +/- SEM of triplicate cultures. C-E, BM-DCs were infected with *H. pylori* PMSS1, PMSS1Δ*ggt* or PMSS1Δ*vacA*; acivicin was added to the infections at 5 µg/ml where indicated. After 16h, bacteria were killed with antibiotics. DCs were co-cultured with immunomagnetically isolated, splenic $CD4^+CD25^-$ T-cells for three days in the presence of rTGF-β, rIL-2 and anti-CD3ε mAb prior to the flow cytometric analysis of CD4, CD25 and FoxP3 expression. Representative plots of the $CD4^+$ gate are shown in C. Pooled results from four (PMSS1Δ*vacA*) to seven (PMSS1Δ*ggt*) independent experiments are shown in D. E, Pooled results from two independent wild type infections performed +/- acivicin; in D and E, each symbol represents one co-culture and horizontal lines indicate the medians. Uninfected DCs and T-cells cultured in the absence of DCs served as controls.

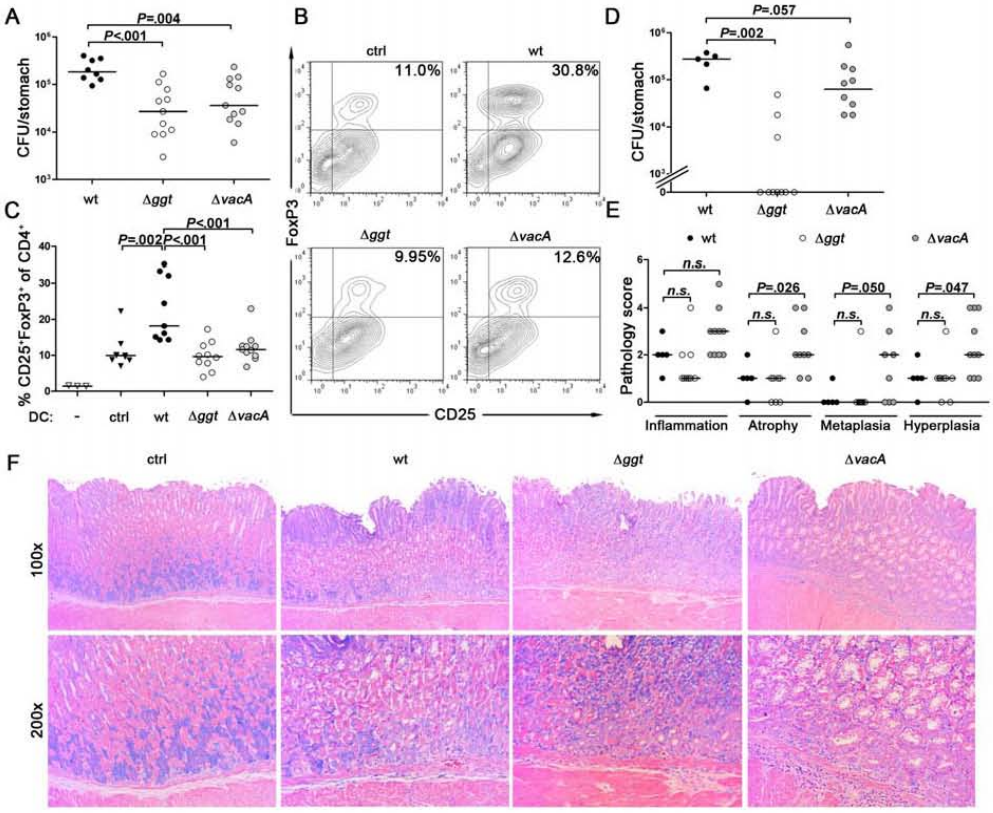


Figure 2. VacA and GGT are required for gastric colonization and DC tolerization *in vivo.* A-C, C57BL/6 mice were infected with *H. pylori* PMSS1, PMSS1Δ*ggt* or PMSS1Δ*vacA* at six weeks of age for one month. Colony forming units per stomach are shown in A. $CD11c^+$ MLN-DCs were immunomagnetically isolated from all infected mice and from uninfected controls, co-cultured with $CD4^+CD25^-$ T-cells, and subjected to flow cytometric analysis of CD4, CD25 and FoxP3 expression. CD25 and FoxP3 staining of the $CD4^+$ gate are shown in B for representative donors. C, Pooled data for all mice shown in A as well as the uninfected donors. Data in A-C are pooled from two independent experiments. D-F, C57BL/6 mice were infected with *H. pylori* PMSS1, PMSS1Δ*ggt* or PMSS1Δ*vacA* at six weeks of age for two months. Colony forming units per stomach are shown in D. E, Pathology scores assigned independently for the parameters inflammation, atrophy, intestinal metaplasia and epithelial hyperplasia. F, Representative micrographs of H&E-stained sections at low (100x) and high magnification (200x). Age-matched uninfected

controls are shown for comparison. In A,C,D and E, each symbol represents one mouse; data in D-F is from one large study.

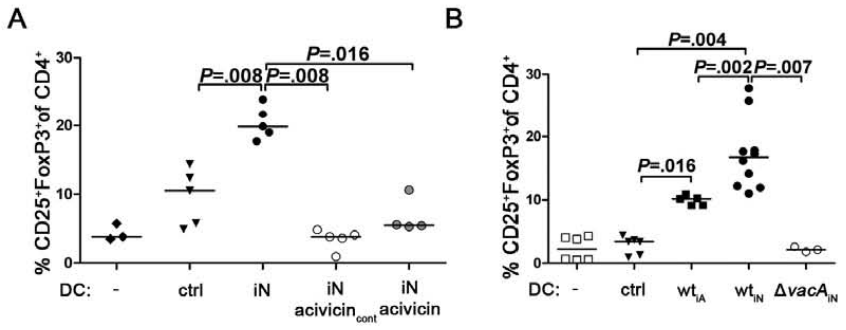


Figure 3. VacA and the enzymatic activity of GGT contribute to neonatally acquired immune tolerance independent of T-cells. A, C57BL/6 mice were infected with *H. pylori* PMSS1 at seven days of age for six weeks. One group received acivicin continuously i.p. every other day at 2 mg/g body weight, starting from the day of infection ($acivicin_{cont}$). Another group received acivicin only during the last two weeks of infection. $CD11c^+$ MLN-DCs were immunomagnetically isolated from all infected mice and from uninfected controls, co-cultured with $CD4^+CD25^-$ T-cells, and stained for CD4, CD25 and FoxP3 expression. CD25 and FoxP3 staining of the $CD4^+$ gate are shown for all co-cultures. B, TCR-$\beta^{-/-}$ mice were infected with *H. pylori* PMSS1 or PMSS1Δ*vacA* at seven days or six weeks of age, for one month. $CD25^+FoxP3^+$ cells in the $CD4^+$ gate are shown for DC/T-cell co-cultures of all donors. Data in B are pooled from two studies.

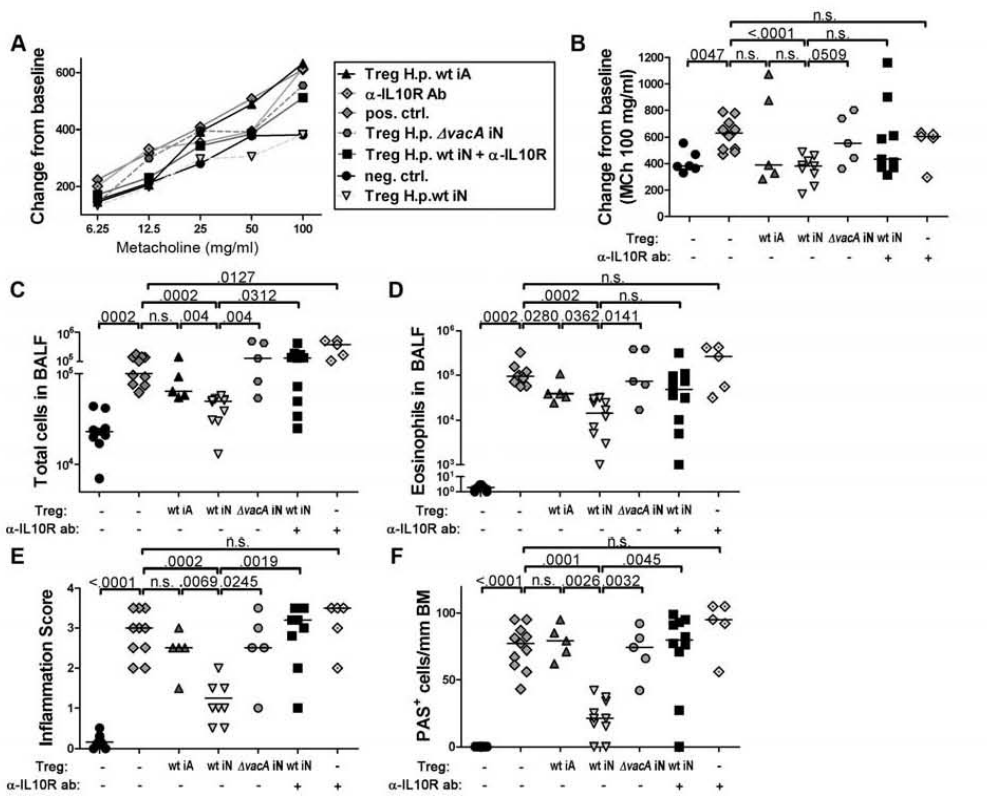


Figure 4. The generation of Tregs with suppressive activity in a model of allergen-induced asthma requires neonatal infection and VacA. A-F, Wild type C57BL/6 mice were sensitized with two i.p. doses of alum-adjuvanted ovalbumin prior to challenge with aerosolized ovalbumin two weeks after the last sensitization. Four groups of sensitized recipients received $CD4^+CD25^+$ MLN T-cells from adult-infected donors (wt iA) or from neonatally wild type- or Δ*vacA*-infected donors (wt iN, Δ*vacA* iN) two days before the first challenge. One of two wt iN Treg recipients groups was further administered two doses of IL-10R neutralizing antibody during the challenge phase (α-IL-10R mAb), along with a positive control group. Negative controls (black circles) were challenged without prior sensitization; positive controls were sensitized and challenged (grey squares). A,B, Airway hyper-responsiveness as assessed with increasing doses of methacholine and with the highest dose of 100 mg/ml. C, Total cells contained in 1ml of BALF. D, Eosinophils in 1ml of BALF.

E, Tissue inflammation as assessed on H&E-stained tissue sections. F, Goblet cell metaplasia as assessed on PAS-stained tissue sections. Data in A-F are pooled from two independent studies.

Supplemental methods

Preparation of murine BM-DCs and MLN-DCs and DC/T-cell co-cultures

For generation of BM-DCs, bone marrow isolated from the hind legs of donor mice was seeded at 50.000 cells per well in 96 well plates in RPMI/10% FCS and 4ng/ml GM-CSF and cultured for 5 days. *E. coli* LPS (Serotype 0111:B4, Sigma-Aldrich) was added at 0.5µg/ml final concentration, unless stated otherwise, to induce BM-DC maturation. For the isolation of MLN-DCs, mesenteric lymph nodes of individual mice were digested in 1mg/ml collagenase (Sigma-Aldrich) for 30 min at 37°C with shaking prior to filtering through a cell strainer (40µm; BD Biosciences) and immunomagnetic isolation of DCs using mouse-specific CD11c microbeads (Miltenyi Biotec). BM-DC cultures were infected o/n with wild type *H. pylori* or the respective *vacA* and *ggt* isogenic mutants at an MOI of 50; bacteria were killed with 200U penicillin/0.2mg streptomycin/ml for 6h prior to the addition of T-cells. $CD4^+CD25^-$ T-cells were prepared from single-cell suspensions of naive C57BL/6 spleens by immunomagnetic sorting (R&D Systems). DCs were co-cultured with $CD4^+CD25^-$ T-cells at a ratio of 1:2 ($0.5x10^5$ DC to$1x10^5$ T-cells) in RPMI containing 10% FCS, 10ng/ml rTGF-β (PeproTech), 10ng/ml rIL-2 (R&D Systems) and 1µg/ml anti-CD3ε (BD Bioscience). After 72h of co-culture, the cells were stained first for CD4 and CD25 and then, after fixation and permeabilization, for FoxP3 (FoxP3-APC, eBioscience). The percentage of $FoxP3^+$ $CD4^+$ T-cells was assessed by FACS. IL12p40 production by DCs was assessed by ELISA (BD Bioscience).

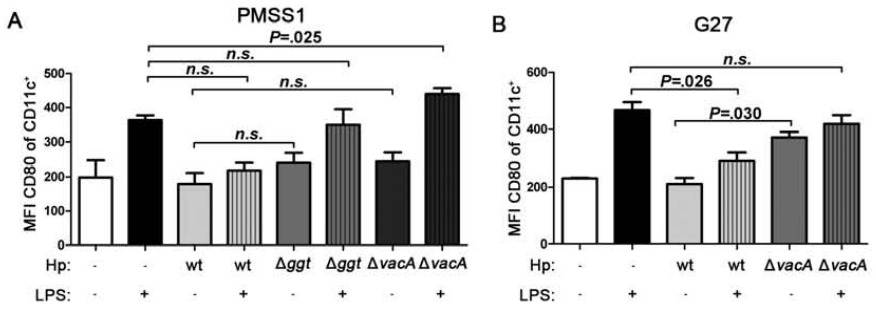
Preparation of murine gastric and mesenteric lymph node single cell suspensions and

One-sixth of every stomach (antrum and corpus) and corresponding mesenteric lymph nodes were digested in 1mg/ml collagenase for 30 min at 37°C with shaking prior to mechanical disruption between glass slides and filtering through a cell strainer (40µm). Single cell suspensions were either seeded at 100.000 cells/well and assessed for IFN-γ and IL-17 production by ELISA (BD Bioscience) or stained directly for FACS analysis. The following antibodies were used: CD11c-biotin, CD4-FITC, CD4-APC (BD Biosciences), CD45-PB and CD80-APC (BioLegend). IFN-γ-PE-Cy7 (BD), IL-17-APC and FoxP3-APC (both eBioscience) were used for intracellular staining. Prior to intracellular cytokine staining, cells were stimulated and blocked in medium containing 2.5µg/ml Brefeldin A (AppliChem), 0.2µM ionomycin (Santa Cruz Biotechnology) and 50ng/ml phorbol 12-myristate 13-acetate (Sigma-Aldrich) for 5h, stained for extracellular markers and fixed in 4% paraformaldehyde. Flow cytometry was performed on a Cyan ADP 9 instrument (Beckman Coulter) and analyzed using FlowJo software (TreeStar).

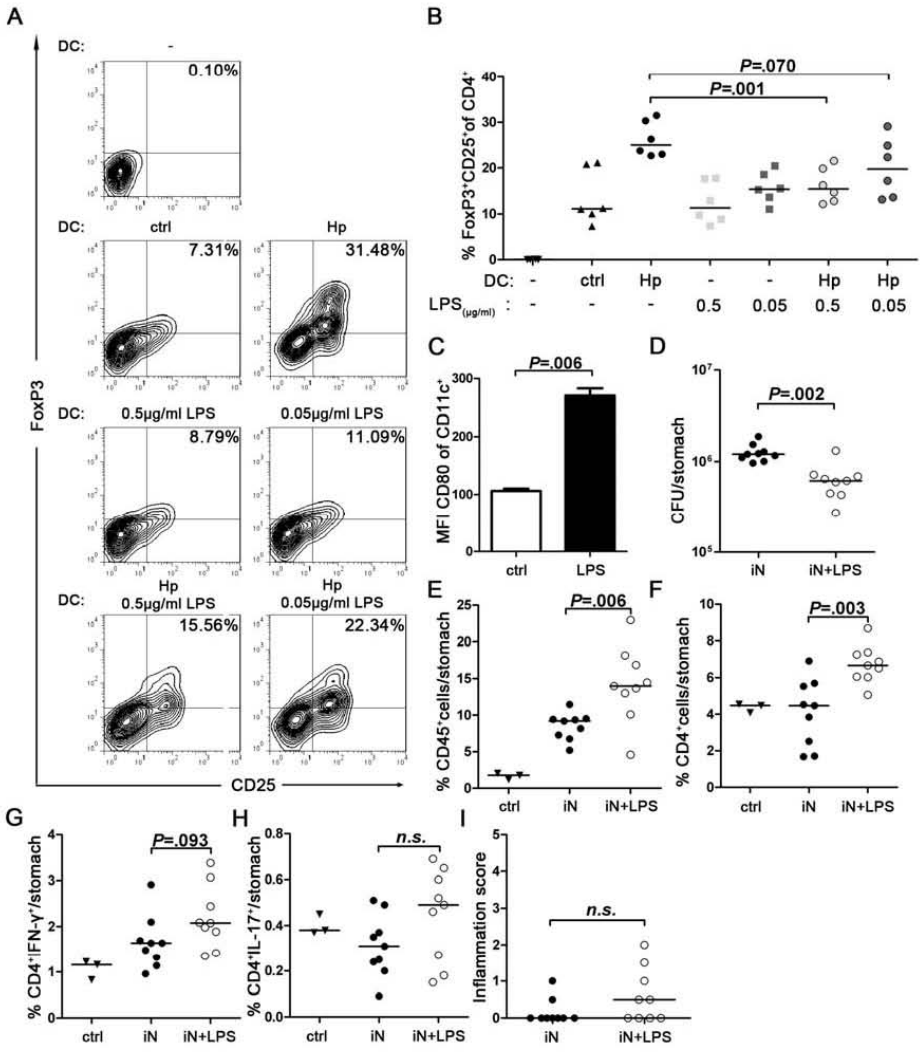
Statistics

GraphPad Prism (GraphPad Software) was used for statistical analyses. All P-values were calculated by Mann-Whitney test unless otherwise indicated. In all scatter plot graphs, the medians are indicated by horizontal bars. In column bar graphs, the SEM is indicated by vertical bars; n.s. stands for 'not significant'. p-values < 0.05 were considered statistically significant.

Supplemental figure legends

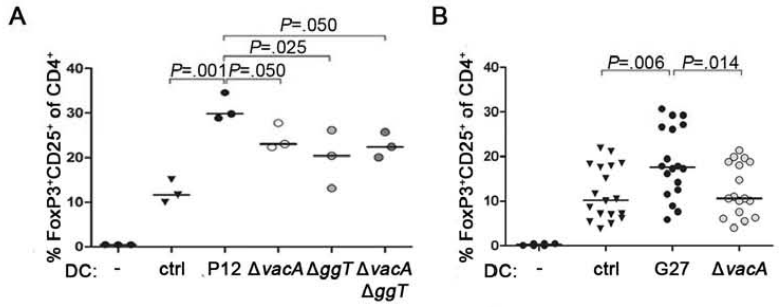


Suppl. Figure 1. VacA and GGT are required for the inhibition of LPS-induced DC maturation by *H. pylori* strains PMSS1 and G27. A) BM-DCs were infected with *H. pylori* strain PMSS1, PMSS1Δ*ggt* and PMSS1Δ*vacA* at a multiplicity of infection (MOI) of 50 and/or treated with 0.5 g/ml *E. coli* LPS for 16h prior to the flow cytometric analysis of CD11c and CD80 expression. B) BM-DCs were infected with *H. pylori* strain G27 and G27Δ*vacA* and/or treated with 0.5µg/ml *E. coli* LPS for 16h prior to the flow cytometric analysis of CD11c and CD80 expression. Data are representative of two to four independent experiments, and are represented as means +/- SEM of triplicate cultures. P-values were calculated using Student's t-test.



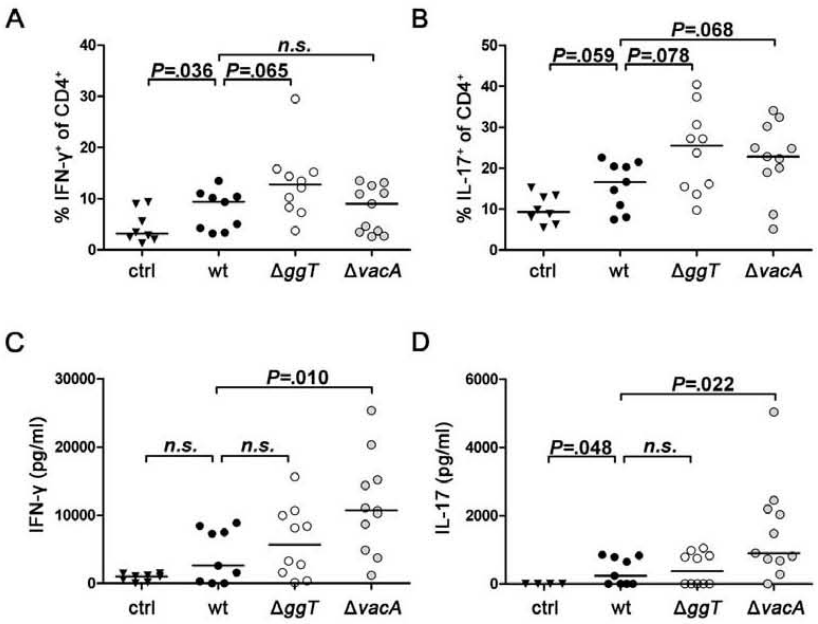
Suppl. Figure 2. *E. coli* LPS impairs *H. pylori*-induced DC tolerization and breaks neonatally acquired immune tolerance to *H. pylori* infection. A, BM-DCs were infected o/n with *H. pylori* PMSS1 (MOI 50) and/or treated with 0.05 or 0.5 µg/ml *E. coli* LPS. After 16h, bacteria were killed with antibiotics. DCs were washed thoroughly and co-cultured with immunomagnetically isolated, splenic $CD4^+CD25^-$ T-cells for three days in the presence of rTGF-β, rIL-2 and anti-CD3ε mAb prior to the flow cytometric analysis of CD4, CD25 and FoxP3 expression. Representative plots of the $CD4^+$ gate are shown. B, Pooled results from two independent infections; each symbol represents one co-culture. C, C57BL/6 mice were

infected with *H. pylori* at seven days (iN) of age for a total of two months. One group received intraperitoneal doses of 1 µg LPS per g of body weight every other day for the final two weeks of infection. MLN single cell suspensions were stained for CD11c and CD80. The mean fluorescence intensity of CD80 expression of CD11c-positive cells is shown for all mice per group. Data are represented as means +/- SEM of triplicate cultures. D, Colony forming units per stomach as determined by plating and colony counting. E, $CD45^+$ leukocyte infiltration into the gastric mucosa of the mice shown in C and D, as well as several uninfected controls. F, $CD4^+$ T-cell infiltration into the gastric mucosa of the mice shown in C-E. G, $CD4^+$IFN- $^+$ T-cell infiltration into the gastric mucosa. H, $CD4^+$IL-17^+ T-cell infiltration into the gastric mucosa. I, Inflammation scores assigned on a scale from 0-6. In C-I, each symbol represents one mouse; data are pooled from two experiments. Horizontal lines indicate the medians. P-values were calculated using the Mann-Whitney test.



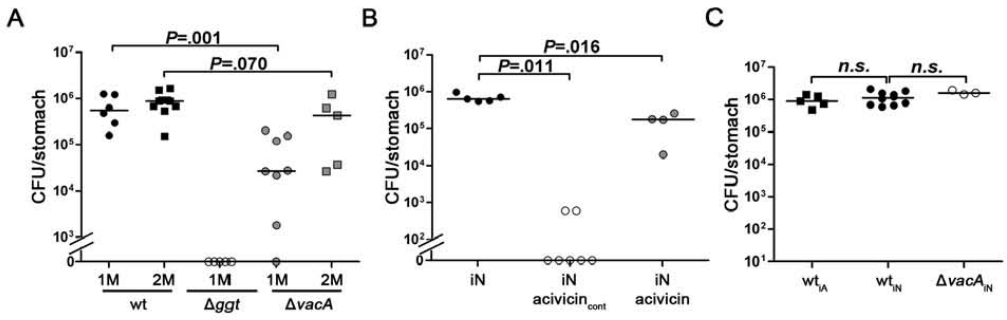
Suppl. Figure 3. VacA and GGT are required for Treg differentiation induced by *H. pylori*-experienced DCs (strains P12 and G27). A) BM-DCs were infected o/n with *H. pylori* strain P12, P12Δ*ggt*, P12Δ*vacA* and P12Δ*ggt*Δ*vacA* at a multiplicity of infection (MOI) of 50. After 16h, bacteria were killed with antibiotics. DCs were co-cultured with

immunomagnetically isolated, splenic $CD4^+CD25^-$ T-cells for three days in the presence of rTGF-β, rIL-2 and anti-CD3ε mAb prior to the flow cytometric analysis of CD4, CD25 and FoxP3 expression. The fraction of $FoxP3^+CD25^+$ cells of the $CD4^+$ gate are shown for triplicate co-cultures. B) BM-DCs were infected o/n with *H. pylori* strain G27 and G27Δ*vacA* at an MOI of 50, and treated as described in A. The fraction of $FoxP3^+CD25^+$ cells of the $CD4^+$ gate are shown for four pooled experiments. Uninfected DCs and T-cells cultured in the absence of DCs served as controls. In A and B, each symbol represents one co-culture. Horizontal lines indicate the medians. P-values were calculated using Student's t-test.



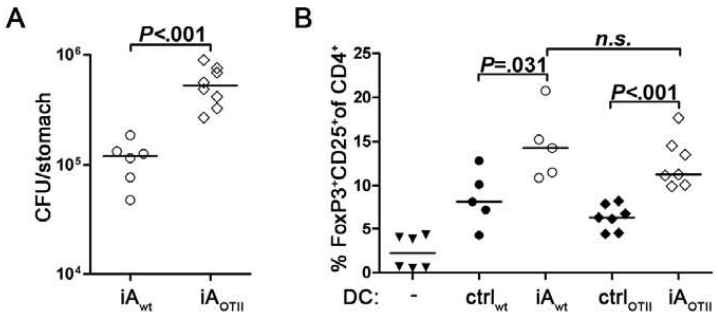
Suppl. Figure 4. VacA and GGT suppress *H. pylori*-specific Th1 and Th17 responses. A) C57BL/6 mice were infected with *H. pylori* PMSS1, PMSS1Δ*ggt* or PMSS1Δ*vacA* at six weeks of age, for one month. Upon sacrifice, MLN single cell preparations of each mouse were stained for CD4 and intracellular IFN-γ. The fraction of IFN-γcells in the $CD4^+$ gate is

shown. B) IL-17/CD4 staining of the same mice as shown in A. C) The same MLN preparations as shown in A and B were seeded at 100.000 cells per well and assessed after three days for IFN- production by ELISA. D) The same supernatants were also subjected to IL-17A ELISAs. Each symbol represents one mouse; horizontal lines indicate medians. P-values were calculated using Student's t-test.

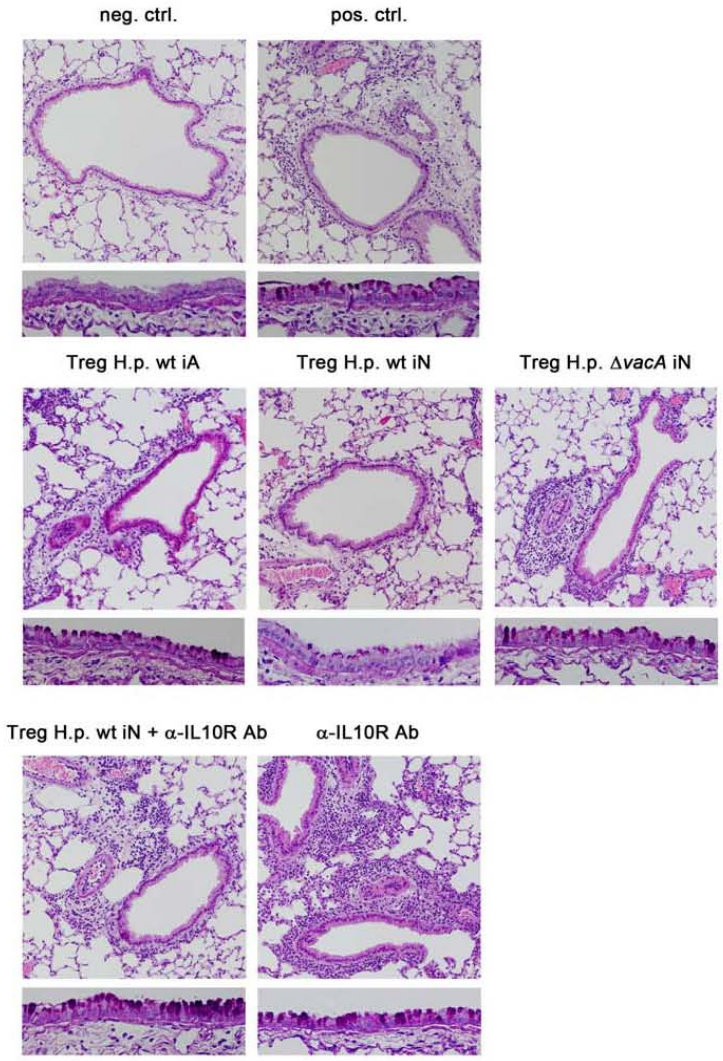


Suppl. Figure 5. VacA and the enzymatic activity of GGT contribute to neonatally acquired immune tolerance independent of T-cells. A, C57BL/6 mice were infected with *H. pylori* PMSS1, PMSS1Δ*ggt* or PMSS1Δ*vacA* at seven days of age, for one or two months. CFU per stomach are shown for all mice in A. B, C57BL/6 mice were infected with *H. pylori* PMSS1 at seven days of age for six weeks. One group received acivicin continuously i.p. every other day at 2 mg/g body weight, starting from the day of infection ($acivicin_{cont}$). Another group received acivicin at the same dosage, but only during the last two weeks of the infection. CFU per stomach are shown in B. C, TCR-$\beta^{-/-}$ mice were infected with *H. pylori* PMSS1 or PMSS1Δ*vacA* at seven days or six weeks of age, for one month. CFU per

stomach are shown in C; horizontal lines in indicate the medians. P-values were calculated using the Mann-Whitney test.



Suppl. Figure 6. A normal T-cell repertoire is not required for the tolerizing effects of VacA- and GGT-proficient *H. pylori* on DCs. A) C57BL/6 or OT II TCR-transgenic mice were infected with *H. pylori* PMSS1 at six weeks of age, for one month. Colony forming units per stomach as determined by plating and colony counting. B) $CD11c^+$ MLN-DCs were immunomagnetically isolated from the mice shown in A and from respective uninfected controls, co-cultured for three days with splenic $CD4^+CD25^-$ T-cells, rTGF-β, rIL-2 and anti-CD3ε mAb, and subjected to flow cytometric analysis of CD4, CD25 and FoxP3 expression. The fraction of $FoxP3^+CD25^+$ cells of the $CD4^+$ gate is shown for all donors shown in A and the uninfected controls. T-cells cultured in the absence of DCs served as additional controls. Data in A and B are from one study. Horizontal lines in indicate the medians. P-values were calculated using the Mann-Whitney test.



Suppl. Figure 7. The generation of Tregs with suppressive activity against lung inflammation and goblet cell metaplasia requires neonatal infection and VacA. Tissue inflammation and goblet cell metaplasia as assessed on H&E and PAS-stained tissue sections. Representative micrographs taken at 100x (H&E) and 400x (PAS) original magnification are shown for representative mice of the groups shown in Figure 7.

7.2 MICRORNA-155 IS ESSENTIAL FOR THE T CELL-MEDIATED CONTROL OF *HELICOBACTER PYLORI* INFECTION AND FOR THE INDUCTION OF CHRONIC GASTRITIS AND COLITIS

Articel published in The Journal of Immunology, 2011

Authors: Mathias Oertli, Daniela B. Engler, Esther Kohler, Manuel Koch, Thomas F. Meyer and Anne Müller

Contributions: I designed, conducted and analyzed all experiments except for figure 1A /3B (MK and TFM), and figure 5 (DBE), EK performed all qRT-PCRs, AM wrote the manuscript

The Journal of Immunology

MicroRNA-155 Is Essential for the T Cell-Mediated Control of *Helicobacter pylori* Infection and for the Induction of Chronic Gastritis and Colitis

Mathias Oertli,*,[1] Daniela B. Engler,*,[1] Esther Kohler,* Manuel Koch,[†] Thomas F. Meyer,[†] and Anne Müller*

MicroRNAs govern immune responses to infectious agents, allergens, and autoantigens and function by posttranscriptional repression of their target genes. In this paper, we have addressed the role of microRNA-155 (miR-155) in the control of *Helicobacter pylori* infection of the gastrointestinal tract and the development of *H. pylori*-induced chronic gastritis and associated gastric preneoplastic pathology. We show that miR-155 is upregulated in the gastric mucosa of experimentally infected mice and that miR-155$^{-/-}$ mice fail to control *H. pylori* infection as a result of impaired pathogen-specific Th1 and Th17 responses. miR-155$^{-/-}$ mice are also less well protected against challenge infection after *H. pylori*-specific vaccination than their wild-type (wt) counterparts. As a consequence of their impaired T cell responses to *H. pylori*, miR-155$^{-/-}$ mice develop less severe infection-induced immunopathology manifesting as chronic atrophic gastritis, epithelial hyperplasia, and intestinal metaplasia. T cells from miR-155$^{-/-}$ mice that are activated by CD3/CD28 cross-linking expand less and produce less IFN-γ and IL-17 than wt T cells. Finally, we show in this paper using adoptive transfers that the phenotypes of miR-155$^{-/-}$ mice are likely due to T cell-intrinsic defects. In contrast to wt T cells, miR-155$^{-/-}$ T cells from infected donors do not control *H. pylori* infections in T cell-deficient recipients, do not differentiate into Th1 or Th17 cells, and do not cause immunopathology. In addition, naive miR-155$^{-/-}$ T cells fail to induce chronic Th17-driven colitis in an adoptive transfer model. In conclusion, miR-155 expression is required for the Th17/Th1 differentiation that underlies immunity to *H. pylori* infection on the one hand and infection-associated immunopathology on the other. *The Journal of Immunology*, 2011, 187: 3578–3586.

MicroRNAs (miRNAs) are an abundant class of small noncoding RNAs that modulate the expression of their target genes at the posttranscriptional level. They bind to the 3′ untranslated regions of specific target mRNAs, thereby suppressing their translation and promoting their degradation. Of the ~700 different miRNAs identified in the human genome to date, at least 100 are expressed in cells of the immune system. The development and function of innate as well as adaptive immune cell populations are now widely believed to be critically dependent on miRNA function (1). One miRNA in particular, miRNA (miR)-155, has received much attention because of its expression and activity in a variety of immune cell types, namely macrophages, dendritic cells (DCs), and various subsets of lymphocytes. In myeloid cells, miR-155 is transcriptionally induced in response to TLR ligands or TNF-α exposure in a manner depending on the transcription factors AP-1 and NF-κB (2). Both pro- and anti-inflammatory effects of miR-155 expression have been reported in myeloid cells, depending on the context and the availability of target mRNAs; proinflammatory effects in myeloid cells have been linked to the repression of negative immune regulators such as SHIP1 and suppressor of cytokine signaling 1 (SOCS1) (3–5), whereas anti-inflammatory effects have been associated with the repression of the signaling protein TAB2 and inhibition of IL-1β expression (6). B cells upregulate miR-155 following their activation in germinal centers; miR-155$^{-/-}$ B cells exhibit defective Ab class switching and fail to differentiate into plasma cells (7, 8). These defects have been attributed to the miR-155–mediated repression of activation-induced deaminase, an enzyme critically involved in class switch recombination and somatic hypermutation (9, 10). T cells in miR-155$^{-/-}$ mice are biased toward Th2 polarization, suggesting that miR-155 promotes Th1 cells (7, 8). A recent study has reported a role for miR-155 in Th17 differentiation and, consequently, found miR-155$^{-/-}$ mice to be highly resistant to experimental autoimmune encephalomyelitis (11).

In this study, we have examined a possible role for miR-155 in controlling experimental infection with the Gram-negative bacterial pathogen *Helicobacter pylori*. Persistent *H. pylori* colonization of its preferred niche, the human gastric mucosa, results in chronic gastritis (12) and predisposes carriers to a high risk of developing gastric and duodenal ulcers, gastric cancer, and gastric MALT lymphoma (13–15). We and others (16–19) have reported earlier that the control of *H. pylori* infection in both experimental infection and vaccination/challenge models requires the activity of Th1 and

*Institute of Molecular Cancer Research, University of Zurich, 8057 Zurich, Switzerland; and [†]Department of Molecular Biology, Max Planck Institute for Infection Biology, 10117 Berlin, Germany

[1]M.O. and D.B.E. contributed equally to this work.

Received for publication June 17, 2011. Accepted for publication July 24, 2011.

This work was supported by grants from the Swiss National Science Foundation, the Cancer League of the Canton of Zurich, and the Foundation for the Fight against Cancer (to A.M.). Additional funding was provided by the University Research Priority Program in Systems Biology/Functional Genomics (to A.M.) and by European Union Grant FP6 LSHG-CT-2006-003900 (SIROCCO) and Deutsche Forschungsgemeinschaft SFB633 (Project B12) (to T.F.M.).

Address correspondence and reprint requests to Prof. Anne Müller, Institute of Molecular Cancer Research, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland. E-mail address: mueller@imcr.uzh.ch

The online version of this article contains supplemental material.

Abbreviations used in this article: BMDC, bone marrow-derived dendritic cell; DC, dendritic cell; DSS, dextran sulfate sodium; miR, microRNA; miRNA, microRNA; PMSS1, premouse Sydney strain 1; qRT-PCR, quantitative RT-PCR; snoR, small nucleolar RNA; SOCS1, suppressor of cytokine signaling 1; Treg, regulatory T cell; wt, wild type.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1101772

Th17 cells. *Helicobacter*-specific Th1 cells and their signature cytokine IFN-γ were further shown to be directly responsible for the induction of infection-associated preneoplastic pathology manifesting as chronic atrophic gastritis, epithelial hyperplasia, and intestinal metaplasia (18, 20). The adoptive transfer of IFN-γ–proficient but not –deficient T cells into TCRβ$^{-/-}$ mice was sufficient to eliminate the infection in the recipients and to induce the full range of preneoplastic pathology (18). Given the major contribution of T cells to the control of—and immunopathology induced by—*H. pylori*, as well as the crucial role of miR-155 in Th1 and Th17 differentiation and the reported upregulation of miR-155 in T cells upon *H. pylori* infection in vitro and in patients (21, 22), we hypothesized that miR-155 should have a clearly discernible role in *H. pylori* pathogenesis. Indeed, we found that miR-155$^{-/-}$ mice were incapable of spontaneously controlling *H. pylori* and of developing vaccine-induced protective immunity and were consequently protected against infection-associated preneoplastic pathology. The phenotype of miR-155–deficient mice could be attributed to a likely T cell-intrinsic defect and was corroborated by the inability of miR-155$^{-/-}$ T cells to proliferate and to produce IFN-γ and IL-17 upon activation via their TCR in vitro.

Materials and Methods

Animal experimentation and H. pylori *cultures*

C57BL/6, Rag1$^{-/-}$ BL6, TCR-β$^{-/-}$ BL6, and miR-155$^{-/-}$ BL6 (B6.Cg-Mir155tm1.1Rsky/J) mice were purchased from The Jackson Laboratory (distributed by Charles River Laboratories, Sulzfeld, Germany). All strains were bred at a University of Zurich specific pathogen-free facility. Mice were maintained in individually ventilated cages and included in studies at 6 wk of age. All animal experimentation was conducted in accordance with cantonal and federal guidelines for the care and use of laboratory animals and was reviewed and approved by the Zurich cantonal veterinary office. Mice were orally immunized three times at weekly intervals with 1 mg *H. pylori* (strain SS1) sonicate adjuvanted with 10 μg cholera toxin (List Biologicals, Campbell, CA) and challenged 1 wk after the last immunization with autologous *H. pylori* by oral gavage of 10^8 bacteria. For adoptive transfer experiments, 300,000 immunomagnetically sorted splenic CD4$^+$CD25$^-$ T cells or naive CD4$^+$CD62L$^+$CD44^{low} T cells (R&D Systems, Minneapolis, MN) were injected into the tail veins of Rag1$^{-/-}$ or TCR-β$^{-/-}$ mice. Dextran sulfate sodium (DSS)-induced colitis was provoked by three cycles of 2% DSS administration in the drinking water (for 5 d) with 7-d compound-free intervals. The *H. pylori* premouse Sydney strain 1 (PMSS1) and its mouse-passaged derivative SS1 as well as agar and liquid culture conditions were described in detail previously (23, 24).

Preparation of gastric/colonic tissue and single-cell suspensions; assessment of H. pylori *colonization and histopathology*

Stomachs and colons were retrieved and dissected longitudinally into equally sized pieces. For quantitative assessment of *H. pylori* colonization, one section of each stomach was homogenized in *Brucella* broth, and serial dilutions were plated on horse blood plates for colony counting. For the quantitative assessment of gastric histopathology, Giemsa-stained paraffin-embedded longitudinal stomach sections ranging from the forestomach/corpus junction to the duodenum were scored independently and in a blinded fashion by two experimenters as described previously (18). Scores on a scale of 0–6 were assigned for the parameters chronic inflammation (in the antrum and corpus areas) as well as corpus atrophy, epithelial hyperplasia, and metaplasia. For the characterization of gastric immune cell infiltrates, one section of every stomach (antrum and corpus) was digested in 1 mg/ml collagenase (Sigma-Aldrich), disrupted between glass slides, filtered through a cell strainer (40 μm; BD Biosciences, San Jose, CA), and processed for flow cytometry (see below). Colonic lamina propria cells were prepared by collagenase digestion, followed by repeated pipetting and processing of single-cell suspensions for flow cytometry. Colon histopathology was scored on Giemsa-stained sections according to the following published guidelines (25): grade 0, no changes observed; grade 1, minimal scattered mucosal inflammatory cell infiltrates, with or without minimal epithelial hyperplasia; grade 2, mild scattered to diffuse inflammatory cell infiltrates, sometimes extending into the submucosa and associated with erosions, with minimal to mild epithelial hyperplasia and minimal to mild mucin depletion from goblet cells; grade 3, mild to moderate inflammatory cell infiltrates that were sometimes transmural, often associated with ulceration, with moderate epithelial hyperplasia and mucin depletion; grade 4, marked inflammatory cell infiltrates that were often transmural and associated with ulceration, with marked epithelial hyperplasia and mucin depletion; and grade 5, marked transmural inflammation with severe ulceration and loss of intestinal glands. Representative images were recorded at ×100 and ×200 final magnification with a Leica Leitz DM RB microscope equipped with a Leica DFC 420C camera. Images were acquired using the Leica Application Suite 3.3.0 software.

Flow cytometry

The following Abs were used for FACS analysis: IFN-γ-PE-Cy7, CD4-FITC (BD Biosciences CA), CD45-Pacific blue, and Ly6-G-allophycocyanin (all BioLegend, San Diego, CA). For intracellular IFN-γ staining, cells were restimulated and blocked for 5 h in medium containing 2.5 μg/ml brefeldin A (AppliChem, Darmstadt, Germany), 0.2 μM ionomycin (Santa Cruz Biotechnology, Santa Cruz, CA), and 50 ng/ml PMA (Sigma-Aldrich). Cells were first stained for CD4 and then fixed in 4% paraformaldehyde and stained for IFN-γ in 0.5% saponin permeabilization buffer. Flow cytometric analysis was performed on a CyanADP instrument and analyzed with FlowJo software (Tree Star, Ashland, OR).

T cell activation assays

CD4$^+$CD25$^-$ T cells were prepared from single-cell suspensions of C57BL/6 wild-type (wt) or miR-155$^{-/-}$ spleens (R&D Systems). T cells were stimulated with anti–CD3/CD28-coated beads (Invitrogen) in the presence of 10 ng/ml rIL-2 (R&D Systems). Bone marrow-derived DCs (BMDCs) were generated by culturing fresh wt bone marrow cells at a density of 50,000 per 96-well flat bottom in RPMI 1640 medium with 10% FCS and 4 ng/ml GM-CSF for 7 d. DCs were matured by addition of 500 ng/ml *Escherichia coli* LPS (Sigma-Aldrich) for 12 h. DCs and T cells were cocultured at a ratio of 1:2 (50,000 DCs:100,000 T cells) in the presence of 1 μg/ml anti-CD3ε (BD Biosciences) and 10 ng/ml rIL-2. After 72 h of coculture, IFN-γ and IL-17 production were quantified by ELISA (R&D Systems) or IFN-γ–specific intracellular cytokine staining for FACS analysis (see flow cytometry). Proliferation was assessed by [^{3}H]thymidine incorporation assay.

Cytokine and miR-155 quantitative RT-PCR

For quantitative RT-PCR (qRT-PCR) of IFN-γ and IL-17, RNA was isolated from scraped gastric mucosa (antrum and corpus) using a NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany). Total RNA (1.5 μg) was used for cDNA synthesis with Superscript Reverse Transcriptase III (Life Technologies). The resulting cDNA served as a template for quantitative PCR performed with a LightCycler 480 using the SYBR Green I Master Kit (Roche, Basel, Switzerland). Absolute values of IFN-γ and IL-17 expression were normalized to GAPDH expression. Primers and conditions were as follows: Tm 55°C, 50 cycles—GAPDH, 5′-GAC ATT GTT GCC ATC AAC GAC C-3′ (forward) and 5′-CCC GTT GAT GAC CAG CTT CC-3′; IFN-γ, 5′-CAT GGC TGT TTC TGG CTG TTA CTG-3′ (forward) and 5′-GTT GCT GAT GGC CTG ATT GTC TTT-3′ (reverse); and IL-17, 5′-GCT CCA GAA GGC CCT CAG A-3′ (forward) and 5′-AGC TTT CCC TCC GCA TTG A-3′. The qRT-PCR for miR-155 quantification was performed according to a two-step manufacturer's protocol (Applied Biosystems) using specific primers for murine miR-155 (Assay-ID 001806) and small nucleolar RNA (snoR)-202 (Assay-ID 001232) and 10 ng total RNA as template.

Statistics

GraphPad Prism 5.0 software (GraphPad, La Jolla, CA) was used for statistical analyses. All p values were calculated by Mann–Whitney U test unless otherwise indicated; Bonferroni–Holm corrections were made for multiple comparisons where appropriate. In all scatter plot graphs, the medians are indicated by horizontal bars. In column bar graphs, the SEM is indicated by vertical bars; n.s. stands for "not significant."

Results

miR-155$^{-/-}$ mice fail to control experimental H. pylori *infection because of their inability to generate Th1 and Th17 responses*

Experimental murine *H. pylori* infections are controlled by MHC class II-restricted, Th1- and Th17-polarized Th cells (16, 18, 24). To examine a possible role for miR-155 in spontaneously reducing

H. pylori colonization upon experimental infection, wt and miR-155$^{-/-}$ mice were infected with the virulent *H. pylori* patient isolate PMSS1 for 1 and 2 mo and analyzed with respect to colonization, gastric cytokine levels, and gastric histopathology (Fig. 1). The expression of miR-155 was induced by ~3-fold in the gastric mucosa of wt mice infected with *H. pylori* for 1 mo (Fig. 1*A*). miR-155$^{-/-}$ mice were colonized by at least 1 order of magnitude more densely than wt animals at both time points (Fig. 1*B*) and exhibited significantly lower levels of gastric IFN-γ and IL-17 production at 2 mo postinfection (Fig. 1*C*, 1*D*). In line with

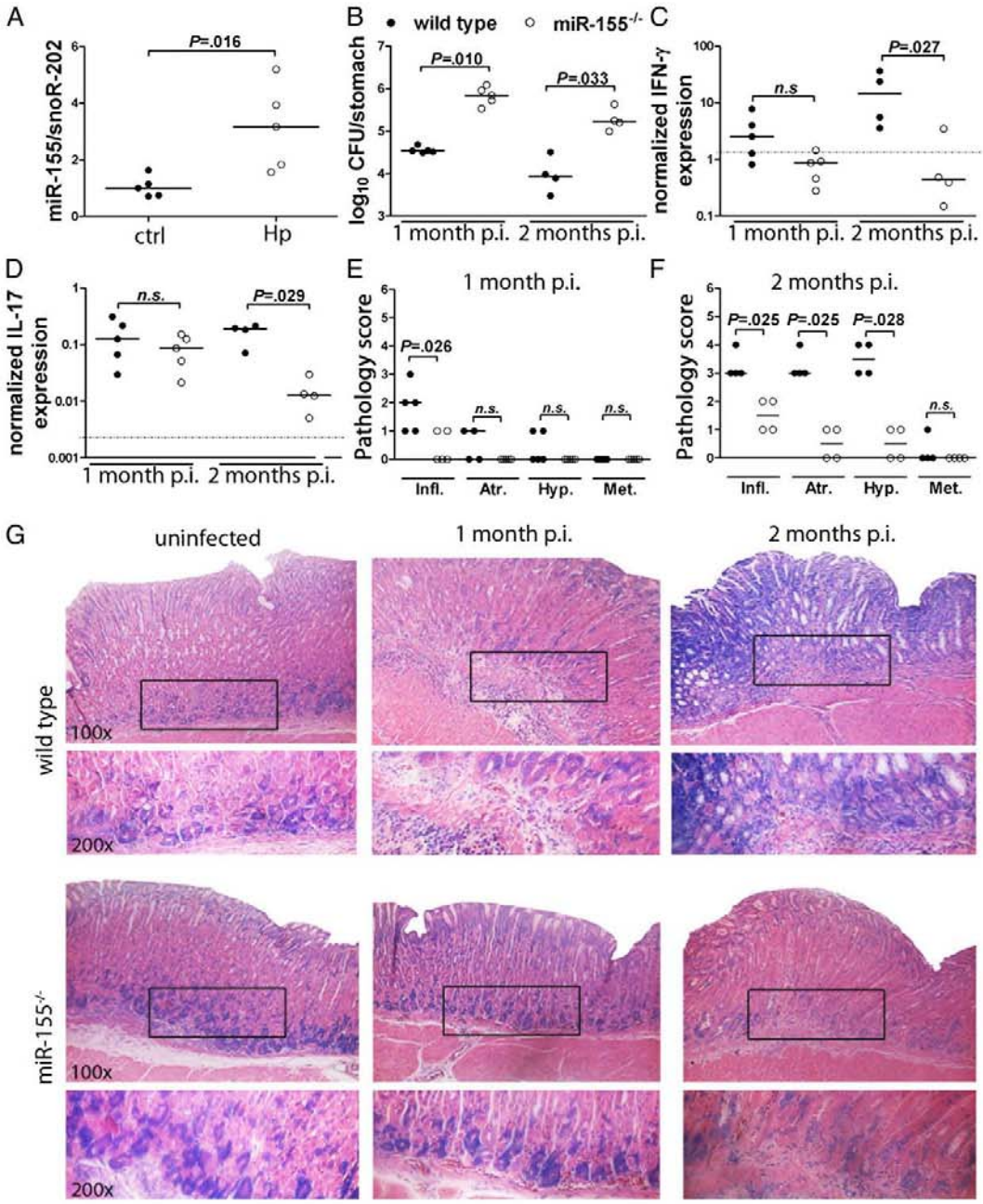


FIGURE 1. miR-155$^{-/-}$ mice are colonized more densely than wt controls, produce less IFN-γ and IL-17, and do not develop preneoplastic gastric pathology. *A*, wt C57BL/6 mice were infected for 1 mo with *H. pylori* PMSS1, and the gastric mucosal expression of miR-155 was assessed by quantitative PCR. miR-155 expression was normalized to snoR-202. *B–G*, C57BL/6 wt and miR-155$^{-/-}$ mice were infected for 1 or 2 mo with *H. pylori* PMSS1 as indicated. *B*, Gastric *H. pylori* colonization as determined by plating and colony counting. *C* and *D*, Gastric mucosal IFN-γ and IL-17 production as determined by qRT-PCR and normalized to GAPDH. The dotted line indicates the average baseline cytokine expression in uninfected controls of both genotypes (three to five mice each). *E–G*, Gastric histopathology at 1 mo (*E*, *G*) and 2 mo (*F*, *G*) postinfection. Scores in *E* and *F* were assigned independently for the parameters inflammation, atrophy, epithelial hyperplasia, and intestinal metaplasia. Representative micrographs of Giemsa-stained sections are shown in *G* at ×100 and ×200 final magnification in relation to uninfected controls of both genotypes. In *A–F*, each data point represents one mouse.

a role for both Th1 and Th17 cells in inducing *H. pylori*-associated gastric inflammation and gastric preneoplastic lesions, miR-155$^{-/-}$ mice were almost completely protected against the histopathological changes manifesting as gastritis, gastric atrophy, epithelial hyperplasia, and intestinal metaplasia that are a hallmark of *H. pylori*-infected wt animals (Fig. 1*E–G*). In conclusion, miR-155 is crucially involved in the generation of Th1 and Th17 responses to experimental *H. pylori* infection, which control the infection on the one hand, and mediate infection-associated gastric immunopathology on the other.

miR-155$^{-/-}$ mice are less well protected than wt mice upon H. pylori*-specific vaccination and challenge*

wt mice that receive three doses of an orally administered whole-cell *H. pylori* vaccine adjuvanted with cholera toxin develop protective immunity against challenge infection with the pathogen (16, 18). To assess whether miR-155$^{-/-}$ mice are protected as well as wt mice, groups of both genotypes were vaccinated three times at weekly intervals prior to autologous challenge infection with the commonly used *H. pylori* vaccine strain SS1. Although both strains were able to reduce *H. pylori* burdens by ~1 order of magnitude as a consequence of their prior immunization, the colonization levels in vaccinated miR-155$^{-/-}$ mice were significantly higher than those of their vaccinated wt counterparts (Fig. 2*A*). The colonization levels of naive (nonvaccinated) mice showed a similar trend (Fig. 2*A*), reproducing the findings obtained by experimental infection with the virulent isolate PMSS1 (Fig. 1*B*). Vaccinated miR-155$^{-/-}$ mice produced lower gastric levels of IFN-γ and IL-17 than vaccinated wt animals (Fig. 2*B*, 2*C*). Their gastric mucosal infiltration of CD45$^+$ leukocytes, a reliable correlate of vaccine-induced protection (16), was significantly reduced compared with wt mice (Fig. 2*D*). As a consequence of their lower cytokine expression and leukocyte infiltration, miR-155$^{-/-}$ mice exhibited less postchallenge gastritis than their vaccinated wt counterparts (Fig. 2*E*). In summary, miR-155 expression is essential for the optimal development of Th1/Th17-driven vaccine-induced protective immunity to *H. pylori* and contributes to the T cell-driven gastritis that is a hallmark of vaccinated, challenged wt mice.

miR-155$^{-/-}$ T cells proliferate less and produce less IFN-γ and IL-17 than wt T cells upon activation in vitro

We and others (16–19) have shown that the spontaneous as well as the vaccine-induced control of *Helicobacter* infection depends on MHC class II-restricted, Th1- and Th17-polarized T cells. Speculating that the T cell-intrinsic expression of miR-155 is required to activate T cells and to generate IFN-γ– and/or IL-17–producing effector cells, we treated immunomagnetically purified CD4$^+$ CD25$^-$ T cells with anti-CD3/anti-CD28 in vitro. In wt T cells, the expression of miR-155 was strongly induced upon anti-CD3/anti-CD28 treatment (Fig. 3*A*). miR-155$^{-/-}$ T cells proliferated less than wt cells upon anti-CD3/anti-CD28 stimulation as determined by thymidine incorporation assay (Fig. 3*B*) and produced significantly less IFN-γ and IL-17 than miR-155–proficient T cells (Fig. 3*C*, 3*D*). In a complementary model, in which LPS-treated BMDCs provided costimulatory signals, miR-155$^{-/-}$ T cells produced less IFN-γ than miR-155–proficient T cells as determined by ELISA and intracellular cytokine staining (Fig. 3*E–G*). IL-17 production was negligible under these circumstances (data not shown). Hypothesizing that regulatory T cells (Tregs) might also rely on miR-155 for proper function, we added immunomagnetically isolated CD4$^+$CD25$^+$ Tregs to the T cell

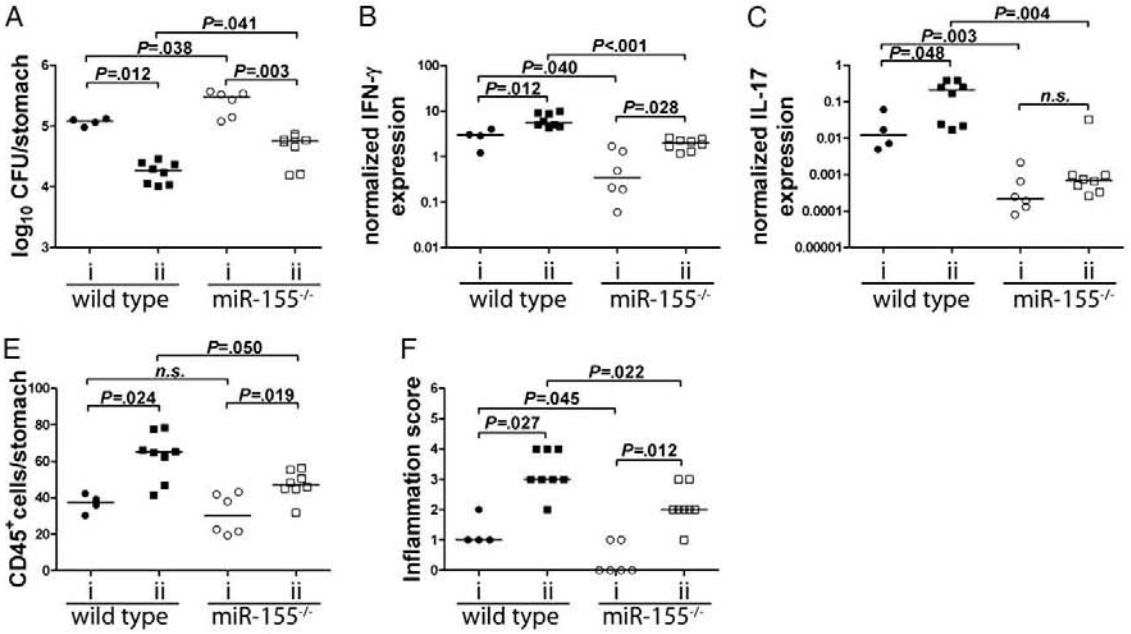


FIGURE 2. Vaccinated miR-155$^{-/-}$ mice are less protected than their wt counterparts upon challenge infection with *H. pylori*. C57BL/6 wt and miR-155$^{-/-}$ mice were vaccinated three times with *H. pylori* SS1 sonicate adjuvanted with cholera toxin prior to challenge infection with the autologous strain. All vaccinated, challenged mice were sacrificed along with naive but challenged controls at 2 wk postchallenge infection. *A*, Gastric *H. pylori* colonization as determined by plating and colony counting. *B* and *C*, Gastric mucosal IFN-γ and IL-17 production as determined by qRT-PCR and normalized to GAPDH. *D*, Gastric mucosal infiltration of CD45$^+$ leukocytes, as determined by flow cytometric analysis of mucosal single-cell preparations. *E*, Inflammation scores as assigned to all mice included in the study. In *A–E*, each data point represents one mouse. i, infected; ii, immunized infected.

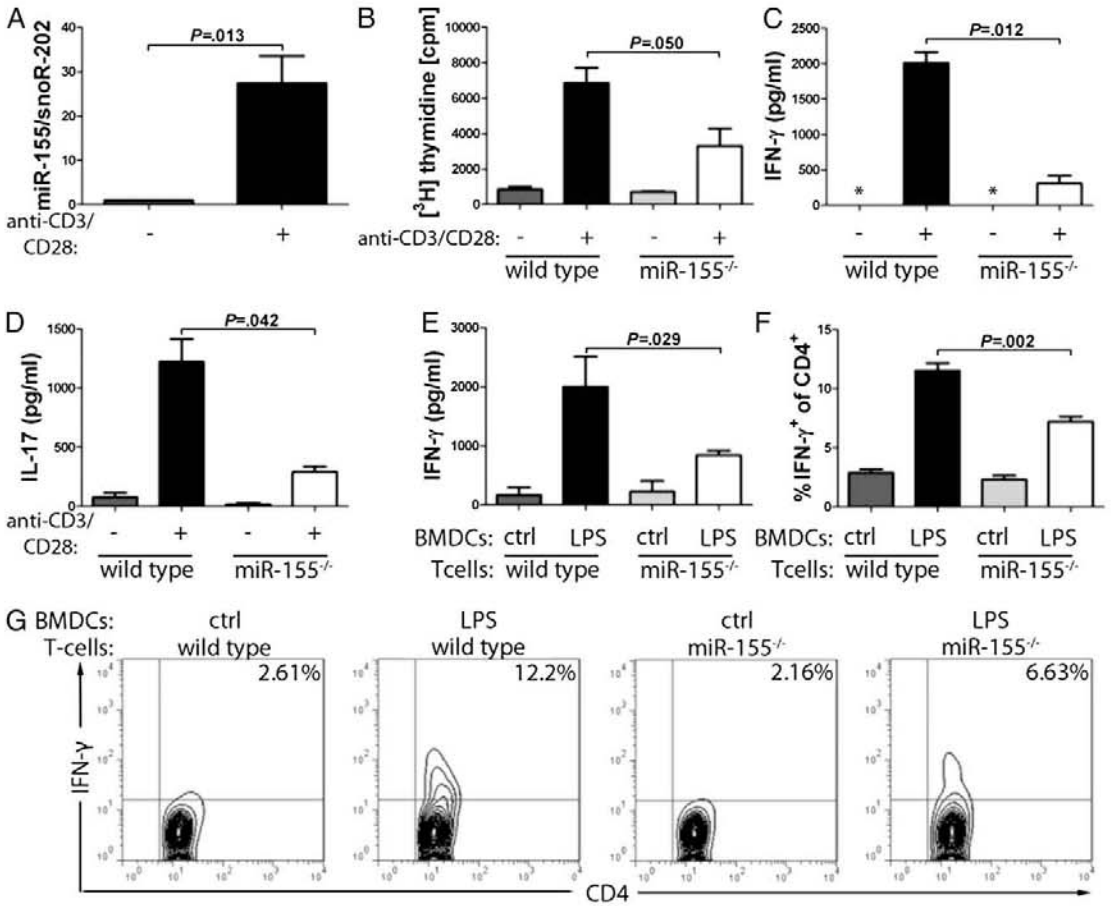


FIGURE 3. miR-155$^{-/-}$ T cells proliferate less and produce less IFN-γ and IL-17 than wt T cells upon activation. *A–D*, wt (and miR-155$^{-/-}$) splenic CD4$^+$CD25$^-$ T cells were treated with anti–CD3/CD28-coated beads for 72 h. *A*, miR-155 expression with and without anti-CD3/CD28 activation as determined by quantitative PCR and normalized to snoR-202. *B*, Proliferation as determined by [^{3}H]thymidine incorporation assay. *C* and *D*, IFN-γ and IL-17 production as quantified by ELISA. *E–G*, BMDCs were activated overnight with 10 μg/ml *E. coli* LPS prior to coculturing with wt or miR-155$^{-/-}$ splenic CD4$^+$CD25$^-$ T cells (1:2 ratio) for 72 h in the presence of anti-CD3 cross-linking Ab. *E*, IFN-γ production as quantified by ELISA. *F* and *G*, IFN-γ expression as determined by intracellular cytokine staining. Representative FACS plots of IFN-γ versus CD4 are shown in *G*; averages of triplicate cocultures are shown in *F*. Vertical bars in *A–F* indicate SEM; *, not detectable. The *p* values were calculated by Student *t* test.

activation system shown in Fig. 3*A–D*. Both wt and miR-155$^{-/-}$ Tregs prevented the CD3/C28 cross-linking–induced proliferation of T cells efficiently (Supplemental Fig. 1), indicating that miR-155 is not required for the suppressive activity of Tregs in vitro. Taken together, the results suggest that miR-155 is essential for the generation of Th1 and Th17 effector T cells subsets but dispensable for Treg function.

miR-155 is required for clearance of H. pylori *and the induction of gastritis and gastric preneoplastic pathology in an adoptive T cell transfer model*

We have shown earlier that the adoptive transfer of purified CD4$^+$ CD25$^-$ T cells from *Helicobacter*-infected donors is sufficient to induce infection-associated gastric preneoplastic pathology in T cell-deficient recipients (18, 24). The development of gastric immunopathology in this model is infection dependent on the part of the donor as well as the recipient (18). To examine a possible effect of miR-155 gene targeting in this model, we infected wt or miR-155$^{-/-}$ donors with *H. pylori* PMSS1 for 1 mo prior to the immunomagnetic isolation of splenic CD4$^+$CD25$^-$ T cells. Rag-1$^{-/-}$ recipients were i.v. injected with either 300,000 wt or miR-155$^{-/-}$ T cells and were experimentally infected on the day of adoptive transfer. Rag-1$^{-/-}$ recipients of miR-155$^{-/-}$ T cells were significantly less capable of clearing the *H. pylori* infection than recipients of wt cells (Fig. 4*A*) and produced lower levels of gastric IFN-γ and IL-17 (Fig. 4*B*, 4*C*). Total leukocyte infiltration into the gastric mucosa was reduced in the recipients of miR-155$^{-/-}$ T cells (Fig. 4*D*), which also exhibited significantly less evidence of gastritis, atrophy, hyperplasia, and metaplasia than the recipients of wt T cells (Fig. 4*E*, 4*F*). In summary, the results suggest that the deficiency of miR-155$^{-/-}$ mice in clearing experimental *H. pylori* infections and in generating vaccine-induced protective immunity is due to a T cell-intrinsic defect. The inability of miR-155$^{-/-}$ T cells to optimally produce IFN-γ or IL-17 upon in vitro activation (Fig. 3) lends further support to this model.

T cell-intrinsic expression of miR-155 is required for the induction of chronic inflammation in a T cell-driven model of colitis

Because of the mechanistic and molecular similarities between *H. pylori*-associated gastritis and T cell-driven models of chronic

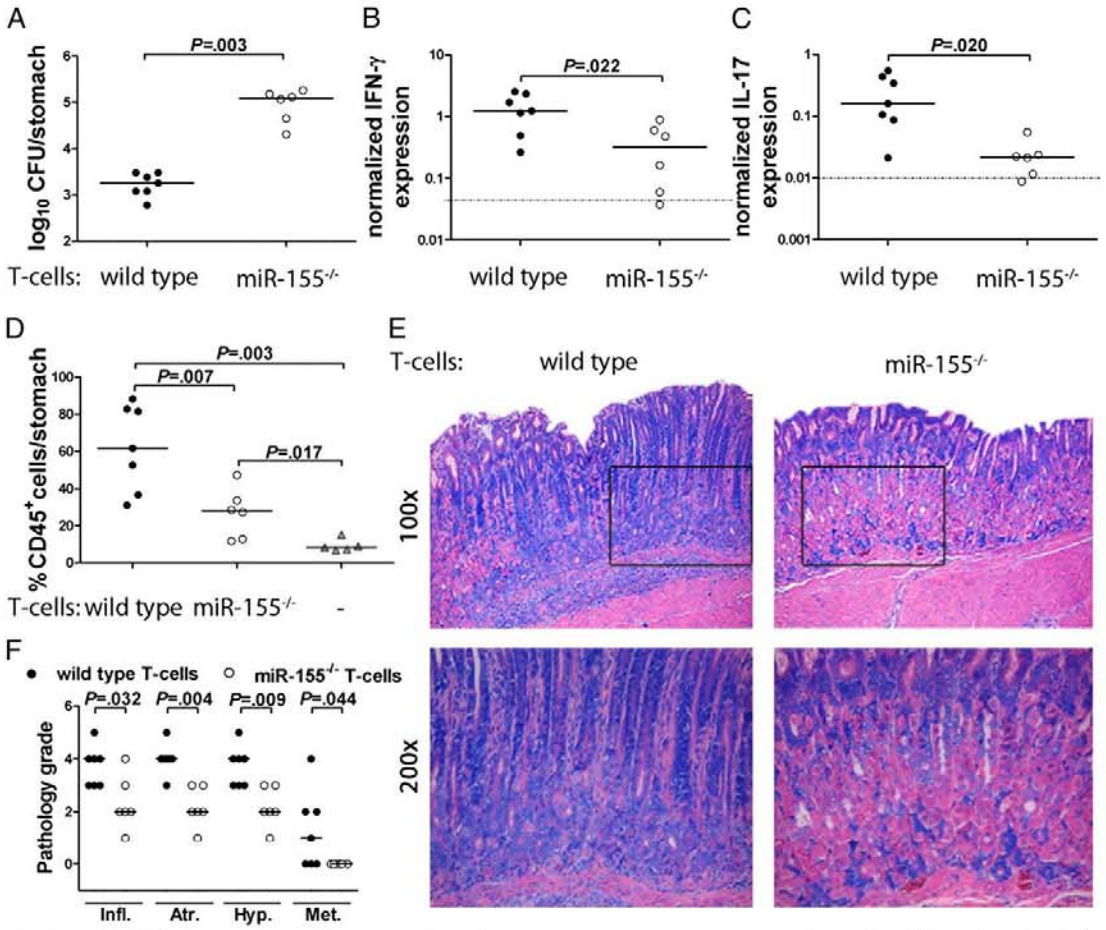


FIGURE 4. miR-155$^{-/-}$ T cells fail to clear *H. pylori* and to induce gastric pathology upon adoptive transfer into Rag-1$^{-/-}$ recipients. Rag-1$^{-/-}$ mice were infected with *H. pylori* PMSS1 and i.v. injected with 300,000 splenic CD4$^+$CD25$^-$ T cells isolated from *H. pylori*-infected wt or miR-155$^{-/-}$ donors. Recipients were sacrificed 1 mo postinfection/adoptive transfer. *A*, Gastric *H. pylori* colonization as determined by plating and colony counting. *B* and *C*, Gastric mucosal IFN-γ and IL-17 production as determined by qRT-PCR and normalized to GAPDH. The dotted line indicates the average baseline cytokine expression in uninfected controls that have not received cells. *D*, Gastric mucosal infiltration of CD45$^+$ leukocytes, as determined by flow cytometric analysis of mucosal single-cell preparations. *E* and *F*, Gastric histopathology as assessed on Giemsa-stained sections; representative micrographs are shown in *E* at ×100 and ×200 final magnification. Scores in *F* were assigned independently for the parameters inflammation, atrophy, epithelial hyperplasia, and intestinal metaplasia. In *A*–*D* and *F*, each data point represents one mouse.

inflammatory conditions of the colon, we hypothesized that the T cell-intrinsic expression of miR-155 should also influence disease outcome in murine colitis models. To test this notion, we adoptively transferred naive wt or miR-155$^{-/-}$ T cells into TCR-β$^{-/-}$ recipients and assessed the development of colitis symptoms 4 wk posttransfer (Fig. 5). The recipients of miR-155$^{-/-}$ T cells exhibited significantly lower colonic expression of IFN-γ and IL-17 than the recipients of wt T cells (Fig. 5*A*, 5*B*). The colonic mucosa of miR-155$^{-/-}$ T cell recipients was further infiltrated by substantially lower numbers of CD45$^+$ leukocytes, CD4$^+$ T cells, and Ly6G$^+$ neutrophils than their wt-recipient counterparts (Fig. 5*C*–*E*). The pathology scores of miR-155$^{-/-}$ T cell recipients—integrating the parameters inflammation, appearance of erosions and ulcerations, and epithelial hyperplasia—were significantly lower than those of wt T cell recipients (Fig. 5*F*, 5*G*). Whereas the latter group presented with on average grade 3 lesions (mild to moderate inflammatory cell infiltrates, sometimes transmural, often associated with ulceration, moderate epithelial hyperplasia, and mucin depletion), only mild, if any, colitis was detected in the recipients of miR-155$^{-/-}$ T cells. In summary, the efficient induction of Th1- and Th17-driven colitis by adoptively transferred, naive T cells requires T cellular miR-155 expression. To assess the susceptibility of miR-155$^{-/-}$ mice in an alternative colitis model, wt and miR-155$^{-/-}$ animals were subjected to three cycles of DSS administration via the drinking water to induce chronic colitis. miR-155$^{-/-}$ mice exhibited lower levels of colonic IFN-γ and IL-17 expression and also developed somewhat milder colitis than wt controls (Supplemental Fig. 2), suggesting that chronic colitis induced by an alternative mechanism also depends at least partially on miR-155.

Discussion

A role for miRNAs in immunity to bacterial infections was initially demonstrated in plants with the discovery that resistance of *Arabidopsis thaliana* to the pathogen *Pseudomonas syringae* depended on miR-393 (26). A first indication that mammalian miRNAs are required for the defense of mammalian cells and tissues against bacterial infectious agents was provided by the finding that

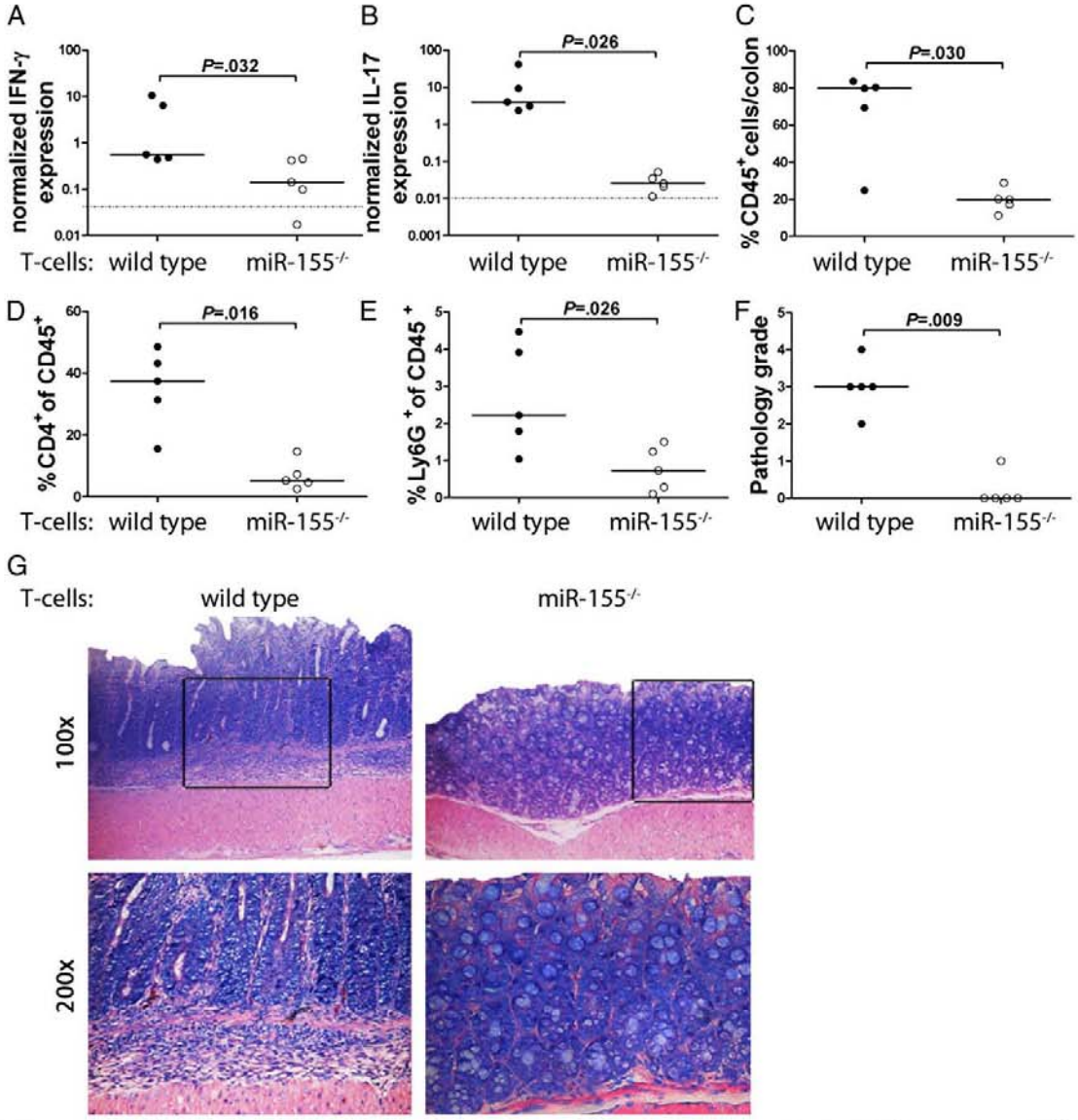


FIGURE 5. miR-155$^{-/-}$ T cells fail to induce IFN-γ and IL-17 and to cause colitis upon adoptive transfer into TCR-β$^{-/-}$ recipients. TCR-β$^{-/-}$ mice were i.v. injected with 300,000 splenic CD4$^+$CD25$^-$ T cells isolated from naive wt or miR-155$^{-/-}$ donors and sacrificed 1 mo postadoptive transfer. *A* and *B*, Colonic mucosal IFN-γ and IL-17 production as determined by qRT-PCR and normalized to GAPDH. The dotted line indicates the average baseline cytokine expression in uninfected controls that have not received cells. *C–E*, Colonic infiltration of CD45$^+$ leukocytes, CD4$^+$ T cells, and Ly6G$^+$ neutrophils as determined by flow cytometric analysis of mucosal single-cell preparations. *F* and *G*, Colonic histopathology as assessed on Giemsa-stained sections; representative micrographs are shown in *G* at ×100 and ×200 final magnification. Scores in *F* were assigned on a scale of 0–5. In *A–F*, each data point represents one mouse.

TLR4 ligands induce the NF-κB–dependent expression of miR-146a/b and miR-155 (2, 4, 6). miR-155$^{-/-}$ mice have since been shown to be hypersusceptible to *Salmonella typhimurium* infection and also cannot be vaccinated protectively against the pathogen (8), despite exhibiting no apparent immunological defects under steady-state conditions. The increased susceptibility of miR-155$^{-/-}$ mice to infectious agents has been attributed to their failure to generate proper Ab responses, which in turn may be due to defective class switching and plasma cell differentiation (7, 8). Additional immune deficiencies of miR-155$^{-/-}$ mice have been linked to DC-intrinsic defects and to imbalanced Th1/Th2 polarization (8). More recent reports using noninfectious disease models have confirmed an involvement of miR-155 in governing Th subset differentiation. Specifically, O'Connell et al. (11) showed that miR-155$^{-/-}$ mice are highly resistant to experimental autoimmune encephalomyelitis because of their defective development and function of Th17 cells.

miR-155 is strongly induced in various cell types upon coculture with *H. pylori* in vitro and is highly elevated in the gastric mucosa of chronically infected patients and of human volunteers experimentally infected with *H. pylori* (21, 22). In gastric epithelial cell lines, *H. pylori* was shown to induce miR-155 expression in an

NF-κB– and AP-1–dependent manner; in *H. pylori*-infected epithelial cells, overexpressed miR-155 appears to downregulate proinflammatory responses such as IL-8 production (22). T cells respond particularly strongly to *H. pylori* exposure (21). We confirm in this study using a model of virulent *H. pylori* infection that miR-155 expression is induced in the gastric mucosa of experimentally infected mice. Naive miR-155–deficient mice are incapable of controlling *H. pylori* and, as a consequence, are protected from the preneoplastic gastric immunopathology that is a hallmark of wt mice infected with virulent *H. pylori* (24). We attribute both the inability of the mice to restrict bacterial growth and their relative lack of pathology to strongly decreased gastric levels of IFN-γ and IL-17. Both cytokines are known to be required for *H. pylori* control and to contribute to gastric preneoplastic pathology (18–20). Interestingly, miR-155$^{-/-}$ mice that have received several doses of an orally administered *H. pylori* vaccine are clearly better able than their nonvaccinated littermates to control a challenge infection. Nevertheless, colonization levels in vaccinated miR-155$^{-/-}$ mice are significantly higher than in vaccinated wt mice, suggesting that optimal protective immunity requires miR-155. Again, decreased gastric levels of IFN-γ and IL-17 in the miR-155$^{-/-}$ mice pointed to defective Th1 and Th17 differentiation and/or function. We propose that T cell-intrinsic effects are responsible for the infection- and vaccination-associated phenotypes of miR-155$^{-/-}$ mice, based on adoptive transfer experiments in which wt but not miR-155$^{-/-}$ T cells effectively cleared the infection in the recipients and at the same time triggered gastric preneoplastic pathology.

Our observation that miR-155$^{-/-}$ T cells proliferate less than wt cells and fail to produce IFN-γ or IL-17 upon CD3/CD28 cross-linking in vitro suggests that miR-155 regulates one or multiple pathways central to T cell activation. Among the targets identified for miR-155 in T cells are the Th1-promoting macrophage-activating factor c-MAF (8) and the negative regulator SOCS1 (5). SOCS1 functions downstream of cytokine receptors and participates in a negative feedback loop to attenuate cytokine signaling. The connection between miR-155 and SOCS1 was initially discovered in Tregs, which require the Foxp3-dependent expression of miR-155 for development (27) and competitive fitness (5). We did not detect a functional defect of miR-155$^{-/-}$ Tregs in an in vitro suppression assay in this study, even though miR-155 is regulated by this Treg-specific transcription factor (21); moreover, the overall phenotype of the miR-155$^{-/-}$ strain is more consistent with a dominant role for miR-155 in effector T cells rather than Tregs in the context of *H. pylori* infection. Elucidating the T cell lineage-specific targets of miR-155 and the relative importance of these targets in Treg and effector T cell functions remains a challenge for future work.

Our T cell adoptive transfer experiments designed to induce *H. pylori* infection-dependent gastritis and infection-independent colitis illustrate the strong defect of miR-155$^{-/-}$ T cells in producing IFN-γ and IL-17 in vivo. The experiments further illustrate the mechanistic similarities of chronic inflammatory conditions of the stomach and lower gastrointestinal tracts, which in both cases are associated with T cellular production of IFN-γ and IL-17. In contrast to O'Connell et al. (11), we do not observe a differential defect in Th17 over Th1 cytokine production, because both cytokines are similarly reduced as a consequence of miR-155 gene deletion in our gastritis and colitis models. Rather, our data point toward a broader and more general role for miR-155 in Th cell differentiation and function. It will be interesting to examine in this context how miR-155$^{-/-}$ mice behave in Th2-driven models of asthma and allergies. In conclusion, the T cell-intrinsic expression of miR-155 is required for the Th1/Th17 cell differentiation and function that underlies immunity to *H. pylori* infection and elicits *Helicobacter* infection-associated immunopathology.

Acknowledgments

We thank Josef Jiricny, Isabelle Arnold, Mara Hartung, Iris Hitzler and Vanessa Craig for helpful discussions. We also thank Alexander Tzankov for advice and instructions on histopathological analysis of colonic inflammation.

Disclosures

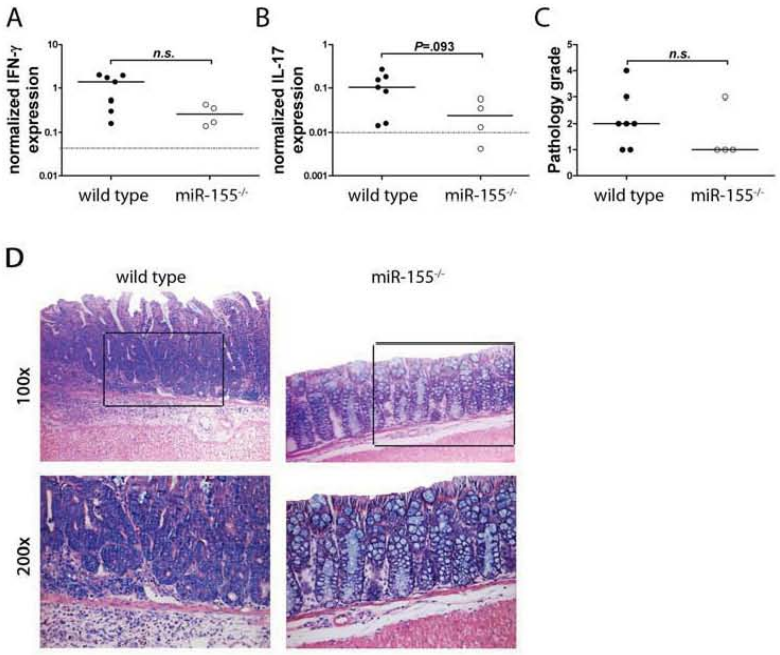
The authors have no financial conflicts of interest.

References

1. O'Connell, R. M., D. S. Rao, A. A. Chaudhuri, and D. Baltimore. 2010. Physiological and pathological roles for microRNAs in the immune system. *Nat. Rev. Immunol.* 10: 111–122.
2. O'Connell, R. M., K. D. Taganov, M. P. Boldin, G. Cheng, and D. Baltimore. 2007. MicroRNA-155 is induced during the macrophage inflammatory response. *Proc. Natl. Acad. Sci. USA* 104: 1604–1609.
3. O'Connell, R. M., A. A. Chaudhuri, D. S. Rao, and D. Baltimore. 2009. Inositol phosphatase SHIP1 is a primary target of miR-155. *Proc. Natl. Acad. Sci. USA* 106: 7113–7118.
4. Androulidaki, A., D. Iliopoulos, A. Arranz, C. Doxaki, S. Schworer, V. Zacharioudaki, A. N. Margioris, P. N. Tsichlis, and C. Tsatsanis. 2009. The kinase Akt1 controls macrophage response to lipopolysaccharide by regulating microRNAs. *Immunity* 31: 220–231.
5. Lu, L. F., T. H. Thai, D. P. Calado, A. Chaudhry, M. Kubo, K. Tanaka, G. B. Loeb, H. Lee, A. Yoshimura, K. Rajewsky, and A. Y. Rudensky. 2009. Foxp3-dependent microRNA155 confers competitive fitness to regulatory T cells by targeting SOCS1 protein. *Immunity* 30: 80–91.
6. Ceppi, M., P. M. Pereira, I. Dunand-Sauthier, E. Barras, W. Reith, M. A. Santos, and P. Pierre. 2009. MicroRNA-155 modulates the interleukin-1 signaling pathway in activated human monocyte-derived dendritic cells. *Proc. Natl. Acad. Sci. USA* 106: 2735–2740.
7. Thai, T. H., D. P. Calado, S. Casola, K. M. Ansel, C. Xiao, Y. Xue, A. Murphy, D. Frendewey, D. Valenzuela, J. L. Kutok, et al. 2007. Regulation of the germinal center response by microRNA-155. *Science* 316: 604–608.
8. Rodriguez, A., E. Vigorito, S. Clare, M. V. Warren, P. Couttet, D. R. Soond, S. van Dongen, R. J. Grocock, P. P. Das, E. A. Miska, et al. 2007. Requirement of bic/microRNA-155 for normal immune function. *Science* 316: 608–611.
9. Teng, G., P. Hakimpour, P. Landgraf, A. Rice, T. Tuschl, R. Casellas, and F. N. Papavasiliou. 2008. MicroRNA-155 is a negative regulator of activation-induced cytidine deaminase. *Immunity* 28: 621–629.
10. Dorsett, Y., K. M. McBride, M. Jankovic, A. Gazumyan, T. H. Thai, D. F. Robbiani, M. Di Virgilio, B. Reina San-Martin, G. Heidkamp, T. A. Schwickert, et al. 2008. MicroRNA-155 suppresses activation-induced cytidine deaminase-mediated Myc-Igh translocation. *Immunity* 28: 630–638.
11. O'Connell, R. M., D. Kahn, W. S. Gibson, J. L. Round, R. L. Scholz, A. A. Chaudhuri, M. E. Kahn, D. S. Rao, and D. Baltimore. 2010. MicroRNA-155 promotes autoimmune inflammation by enhancing inflammatory T cell development. *Immunity* 33: 607–619.
12. Marshall, B. J., and J. R. Warren. 1984. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet* 1: 1311–1315.
13. Parsonnet, J., G. D. Friedman, D. P. Vandersteen, Y. Chang, J. H. Vogelman, N. Orentreich, and R. K. Sibley. 1991. *Helicobacter pylori* infection and the risk of gastric carcinoma. *N. Engl. J. Med.* 325: 1127–1131.
14. Parsonnet, J., S. Hansen, L. Rodriguez, A. B. Gelb, R. A. Warnke, E. Jellum, N. Orentreich, J. H. Vogelman, and G. D. Friedman. 1994. *Helicobacter pylori* infection and gastric lymphoma. *N. Engl. J. Med.* 330: 1267–1271.
15. Pritchard, D. M., and J. E. Crabtree. 2006. *Helicobacter pylori* and gastric cancer. *Curr. Opin. Gastroenterol.* 22: 620–625.
16. Hitzler, I., M. Oerth, B. Becher, E. M. Agger, and A. Muller. 2011. Dendritic cells prevent rather than promote immunity conferred by a *Helicobacter* vaccine using a mycobacterial adjuvant. *Gastroenterology* 141: 186–196.e1.
17. Akhiani, A. A., J. Pappo, Z. Kabok, K. Schön, W. Gao, L. E. Franzén, and N. Lycke. 2002. Protection against *Helicobacter pylori* infection following immunization is IL-12-dependent and mediated by Th1 cells. *J. Immunol.* 169: 6977–6984.
18. Sayi, A., E. Kohler, I. Hitzler, I. Arnold, R. Schwendener, H. Rehrauer, and A. Müller. 2009. The CD4$^+$ T cell-mediated IFN-γ response to *Helicobacter* infection is essential for clearance and determines gastric cancer risk. *J. Immunol.* 182: 7085–7101.
19. Velin, D., L. Favre, E. Bernasconi, D. Bachmann, C. Pythoud, E. Saiji, H. Bouzourene, and P. Michetti. 2009. Interleukin-17 is a critical mediator of vaccine-induced reduction of *Helicobacter* infection in the mouse model. *Gastroenterology* 136: 2237–2246.e1.
20. Stoicov, C., X. Fan, J. H. Liu, G. Bowen, M. Whary, E. Kurt-Jones, and J. Houghton. 2009. T-bet knockout prevents *Helicobacter* felis-induced gastric cancer. *J. Immunol.* 183: 642–649.
21. Fassi Fehri, L., M. Koch, E. Belogolova, H. Khalil, C. Bolz, B. Kalali, H. J. Mollenkopf, M. Beigier-Bompadre, A. Karlas, T. Schneider, et al. 2010.

Helicobacter pylori induces miR-155 in T cells in a cAMP-Foxp3-dependent manner. *PLoS One* 5: e9500.
22. Xiao, B., Z. Liu, B. S. Li, B. Tang, W. Li, G. Guo, Y. Shi, F. Wang, Y. Wu, W. D. Tong, et al. 2009. Induction of microRNA-155 during *Helicobacter pylori* infection and its negative regulatory role in the inflammatory response. *J. Infect. Dis.* 200: 916–925.
23. Lee, A., J. O'Rourke, M. C. De Ungria, B. Robertson, G. Daskalopoulos, and M. F. Dixon. 1997. A standardized mouse model of *Helicobacter pylori* infection: introducing the Sydney strain. *Gastroenterology* 112: 1386–1397.
24. Arnold, I. C., J. Y. Lee, M. R. Amieva, A. Roers, R. A. Flavell, T. Sparwasser, and A. Müller. 2011. Tolerance rather than immunity protects from *Helicobacter pylori*-induced gastric preneoplasia. *Gastroenterology* 140: 199–209.
25. Asseman, C., S. Mauze, M. W. Leach, R. L. Coffman, and F. Powrie. 1999. An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. *J. Exp. Med.* 190: 995–1004.
26. Navarro, L., P. Dunoyer, F. Jay, B. Arnold, N. Dharmasiri, M. Estelle, O. Voinnet, and J. D. Jones. 2006. A plant miRNA contributes to antibacterial resistance by repressing auxin signaling. *Science* 312: 436–439.
27. Kohlhaas, S., O. A. Garden, C. Scudamore, M. Turner, K. Okkenhaug, and E. Vigorito. 2009. Cutting edge: the Foxp3 target miR-155 contributes to the development of regulatory T cells. *J. Immunol.* 182: 2578–2582.

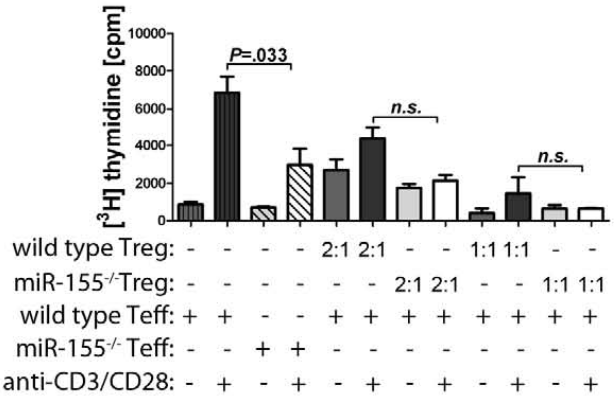
Suppl. Fig. 2:



Suppl. Fig. 2. *miR-155$^{-/-}$ mice develop less severe colitis than wild type controls.* miR-155$^{-/-}$ and wild type animals were subjected to three cycles of DSS administration via the drinking water. *A,B,* Colonic mucosal IFN-γ and IL-17 production as determined by qRT-PCR, normalized to GAPDH. The dotted line indicates the average baseline cytokine expression in untreated controls. *C,D,* Colonic histopathology as assessed on Giemsa-stained sections; representative micrographs are shown in D at 100x and 200x final magnification. Scores in C were assigned on a scale of 0-5. In A-C, each data point represents one mouse.

Supplemental Figures

Suppl. Fig. 1:



Suppl. Fig. 1. *miR-155 is not required for the suppressive activity of Tregs* in vitro. Wild type and miR155$^{-/-}$ splenic CD4^{+}CD25^{-} T-cells were treated with anti-CD3/CD28-coated beads for 72 hours ore left untreated. Wild type or miR155$^{-/-}$ splenic CD4^{+}CD25^{+} Tregs were added at a 2:1 (100.000 CD4^{+}CD25^{-} T-cells to 50.000 Tregs) or 1:1 ratio (100.000 CD4^{+}CD25^{-} T-cells to 100.000 Tregs) as indicated. The proliferation was determined by [^{3}H] thymidine incorporation assay.

7.3 THE C-TERMINALLY ENCODED, MHC CLASS II-RESTRICTED T CELL ANTIGENICITY OF THE *HELICOBACTER PYLORI* VIRULENCE FACTOR CAGA PROMOTES GASTRIC PRENEOPLASIA

Article published in The Journal of Immunology, 2011

Authors: Isabelle C. Arnold, Iris Hitzler, Daniela B. Engler, Mathias Oertli, Else-Marie Agger and Anne Müller

Contributions: I helped to perform and analyze the TCR-$\beta^{-/-}$ knock-out experiments and the tolarization experiments by CD40/CD40L modulation. IA designed performed and analyzed the study with help by DE and IH, AM wrote the manuscript. EMA provided reagents.

The Journal of Immunology

The C-Terminally Encoded, MHC Class II-Restricted T Cell Antigenicity of the *Helicobacter pylori* Virulence Factor CagA Promotes Gastric Preneoplasia

Isabelle C. Arnold,* Iris Hitzler,* Daniela Engler,* Mathias Oertli,* Else Marie Agger,† and Anne Müller*

Chronic infection with the human bacterial pathogen *Helicobacter pylori* causes gastritis and predisposes carriers to an increased gastric cancer risk. Consequently, *H. pylori*-specific vaccination is widely viewed as a promising strategy of gastric cancer prevention. *H. pylori* strains harboring the Cag pathogenicity island (PAI) are associated with particularly unfavorable disease outcomes in humans and experimental rodent models. We show in this study using a C57BL/6 mouse model of Cag-PAI$^+$ *H. pylori* infection that the only known protein substrate of the Cag-PAI–encoded type IV secretion system, the cytotoxin-associated gene A (CagA) protein, harbors MHC class II-restricted T cell epitopes. Several distinct nonoverlapping epitopes in CagA's central and C-terminal regions were predicted in silico and could be confirmed experimentally. CagA$^+$ infection elicits CD4$^+$ T cell responses in mice, which are strongly enhanced by prior mucosal or parenteral vaccination with recombinant CagA. The adoptive transfer of CagA-specific T cells to T cell-deficient, *H. pylori*-infected recipients is sufficient to induce the full range of preneoplastic immunopathology. Similarly, immunization with a cholera toxin-adjuvanted, CagA$^+$ whole-cell sonicate vaccine sensitizes mice to, rather than protects them from, *H. pylori*-associated gastric cancer precursor lesions. In contrast, *H. pylori*-specific tolerization by neonatal administration of *H. pylori* sonicate in conjunction with a CD40L-neutralizing Ab prevents *H. pylori*-specific, pathogenic T cell responses and gastric immunopathology. We conclude that active tolerization may be superior to vaccination strategies in gastric cancer prevention. *The Journal of Immunology*, 2011, 186: 6165–6172.

Persistent gastric infection with the bacterial pathogen *Helicobacter pylori* results in chronic gastritis (1) and predisposes carriers to a high risk for development of gastric and duodenal ulcers, gastric cancer, and gastric MALT lymphoma (2–4). Despite a recent decline in infection rates in industrialized countries, the prevalence rate of *H. pylori* remains at nearly 100% in the developing world (5). The infection can be eradicated in a majority of individuals by antibiotic therapy; however, resistance rates are rising (6), and vaccination against *H. pylori* is viewed as a cost-effective alternative to eradication therapy (7). Several vaccination regimens induce protective immunity in animal models; these include subunit vaccines containing *H. pylori* urease (8), neutrophil-activating protein (9), or adhesin A (10), but also recombinant live *Salmonella* vaccines expressing *Helicobacter* Ags (11). Clinical trials have demonstrated the immunogenicity of experimental vaccines in human volunteers (12–14). However, all current vaccine development efforts are hampered by their failure to achieve sterilizing immunity in rodent models, let alone in humans.

Helicobacter strains harboring the cytotoxin-associated gene A-encoded virulence factor CagA have been associated with high levels of gastric inflammation (15) and an increased gastric cancer risk compared with CagA$^-$ strains (16, 17). CagA is the only known protein substrate of a pathogenicity island-encoded type IV secretion system (T4SS), which allows the bacteria to deliver the virulence factor directly into their host cell's cytosol (18). Upon injection, CagA is tyrosine phosphorylated on C-terminal motifs, leading to the loss of cell-to-cell contacts, cell scattering, and increased motility (19). CagA delivery to the host cell further disrupts cell polarity (20) and allows the bacteria to colonize the apical surface of cultured cells (21). In vivo, transgenic expression of CagA under a stomach-specific promoter is by itself sufficient to induce epithelial hyperplasia and, in a subset of mice, gastric polyps and adenocarcinoma (22), implying that CagA can function as a bacterial oncoprotein. In a Mongolian gerbil model of CagA$^+$ *H. pylori* infection, the bacteria induce gastric cancer precursor lesions that resemble *H. pylori*-associated lesions in humans (23). We have recently introduced a C57BL/6 mouse model of infection with a Cag$^+$ *H. pylori* patient isolate that induces atrophic gastritis, epithelial hyperplasia, and intestinal metaplasia in its host in a T4SS-dependent manner (24). In this model, the age of the host at the time of infection determines disease outcome. Mice that are experimentally infected during the neonatal period develop immunological tolerance rather than immunity to *H. pylori* and are protected from the immunopathological T cell responses that are a hallmark of CagA$^+$ infection in adults (24).

*Institute of Molecular Cancer Research, University of Zurich, 8057 Zurich, Switzerland; and †Department of Infectious Disease Immunology, Statens Serum Institute, 2300 Copenhagen, Denmark

Received for publication October 20, 2010. Accepted for publication March 30, 2011.

This work was supported by grants from the Swiss National Science Foundation (to A.M.), the Swiss Cancer League (to A.M.), the Cancer League of the Canton of Zurich (to A.M.), and the University Research Priority Program in Systems Biology/Functional Genomics.

Address correspondence and reprint requests to Prof. Anne Müller, Institute of Molecular Cancer Research, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland. E-mail address: mueller@imcr.uzh.ch

The online version of this article contains supplemental material.

Abbreviations used in this article: CagA, cytotoxin-associated gene A; CT, cholera toxin; DC, dendritic cell; MLN, mesenteric lymph node; PAI, pathogenicity island; p.i., postinfection; PMSS1, premouse Sydney strain 1; rCagA, recombinant CagA; T4SS, type IV secretion system.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1003472

In this study, we pursued the hypothesis that the CagA protein itself functions as a "pathogenic," procarcinogenic T cell Ag. By analyzing the antigenicity of recombinantly expressed full-length CagA, as well as truncated fragments and in silico-predicted CagA peptides, we have identified several distinct MHC class II-restricted T cell epitopes in the central and C-terminal regions of the protein. We further show in this article that immunization with CagA, either systemically or mucosally, greatly enhances the host's T cell response to challenge infection but fails to afford protective immunity. Immunization with a CagA$^+$ whole-cell sonicate vaccine sensitizes mice to, rather than protects them from, gastric cancer precursor lesions. In contrast, active tolerization of neonatal mice by administration of *H. pylori* sonicate in conjunction with a CD40L blocking Ab before experimental infection prevents pathogenic T cell responses and protects mice from preneoplastic gastric changes. We conclude that tolerization strategies may hold more promise than vaccination for the prevention and management of *H. pylori*-associated gastric disease manifestations.

Materials and Methods

Animal experimentation and H. pylori *cultures*

C57BL/6 and TCR-$\beta^{-/-}$ BL6 mice were purchased from Charles River Laboratories (Sulzfeld, Germany) and bred at a University of Zurich specific pathogen-free facility. Mice were maintained in individually ventilated cages and included in studies at 6 wk of age. All animal experimentation was conducted in accordance with cantonal and federal guidelines for the care and use of laboratory animals, and was reviewed and approved by the Zurich cantonal veterinary office (permit number 63/2008 to A.M.). Mice were immunized four times at weekly intervals with 100 μg recombinant CagA (rCagA) or 1 mg *H. pylori* (premouse Sydney strain 1 [PMSS1]) sonicate, either adjuvanted with 10 μg cholera toxin (CT; List Biologicals, Campbell, CA) and given orally, or administered s.c. with CAF01. A stable CAF01 formulation consisting of dimethyldioctadecylammonium bromide and a,a′-trehalose 6,6′-dibehenate (Avanti Polar Lipids) was prepared by the lipid film hydration method as previously described (25). All mice were challenged 1 wk after the last immunization with *H. pylori* PMSS1 by oral gavage of 10^8 bacteria. Anti-CD40L mAb (clone MR1) and anti-CD40 mAb (clone FGK 4.5; both Bio X Cell, West Lebanon, PA) were i.p. injected at 50 or 75 μg/dose into neonatal mice on days 7, 10, 12, and 14 after birth. Neonatal mice were infected at 7 d of age with 2×10^7 CFU *H. pylori* PMSS1 in 50 μl. For adoptive transfer experiments, 300,000 immunomagnetically sorted splenic CD4$^+$CD25$^-$ T cells (CD4$^+$CD25$^+$ T cell purification kit; R&D Systems, Minneapolis, MN) were injected into the tail veins of TCR-$\beta^{-/-}$ mice. The *H. pylori* PMSS1 used in this study, as well as agar and liquid culture conditions, was described in detail previously (24).

Preparation of gastric tissue and gastric/mesenteric lymph node single-cell suspensions: assessment of H. pylori *colonization, histopathology, and IFN-γ production*

Stomachs were retrieved and dissected longitudinally into equally sized pieces. For quantitative assessment of *H. pylori* colonization, one section of each stomach was homogenized in *Brucella* broth, and serial dilutions were plated on horse blood plates for colony counting. For the quantitative assessment of gastric histopathology, Giemsa-stained, paraffin-embedded, longitudinal stomach sections ranging from the forestomach/corpus junction all the way to the duodenum were scored independently and in a blinded fashion by two experimenters. Scores on a scale of 0–6 were assigned for the parameters' chronic inflammation (in the antrum and corpus areas), as well as corpus atrophy, epithelial hyperplasia, and metaplasia. For the characterization of gastric immune cell infiltrates, one sixth of every stomach (antrum and corpus) was digested in 1 mg/ml collagenase (Sigma-Aldrich), disrupted between glass slides, filtered through a cell strainer (40 μm; BD Biosciences, San Jose, CA), and processed for flow cytometry (see later). Mesenteric lymph nodes (MLN) of individual mice were disrupted by collagenase digestion followed by repeated pipetting and filtering; single-cell suspensions were either subjected to immunomagnetic cell isolation (CD4$^+$CD25$^+$ T cell purification kit; R&D Systems) or cultured for 4 d in round-bottom 96-well plates for assessment of IFN-γ secretion by ELISA (BD Biosciences) or by intracellular cytokine staining (see later).

Flow cytometry

The following Abs were used for FACS analysis: IFN-γ–PE–Cy7, CD4-FITC (BD Biosciences, San Jose, CA), CD45-PB, CD62L-allophycocyanin, CD44-PB, GR-1 Ly6-G–allophycocyanin, and rat anti-mouse c-Kit/CD117 (all from BioLegend, San Diego, CA). For intracellular IFN-γ staining, cells were restimulated and blocked for 5 h in medium containing 2.5 μg/ml brefeldin A (AppliChem, Darmstadt, Germany), 0.2 μM ionomycin (Santa Cruz Biotechnology, Santa Cruz, CA), and 50 ng/ml phorbol 12-myristate 13-acetate (Sigma-Aldrich). Cells were first stained for CD4 and then fixed in 4% paraformaldehyde and stained for IFN-γ in 10% saponin permeabilization buffer. Flow cytometric analysis was performed on a CyanADP instrument and analyzed with Summit software (Beckman Coulter, Brea, CA).

Generation of rCagA fragments and synthetic peptides, and assessment of CagA-specific T cell responses

For the generation of full-length rCagA and its fragments, PCR products were obtained with HotStar High Fidelity DNA Polymerase (Qiagen) and cloned into the BamH1 and SalI restriction sites of the pGEX 4T3 expression vector with an N-terminal GST-tag. All primer sequences and PCR conditions are listed in Supplemental Table I. Protein expression was induced in *Escherichia coli* BL21 by isopropyl β-D-thiogalactopyranoside for 4 h at 27°C, followed by glutathione Sepharose affinity chromatography. MHC class II-binding 15-aa peptides were predicted with Rankpep (http://immunax.dfci.harvard.edu/Tools/rankpep.html) and synthesized by Thermo Fisher Scientific (peptide 11: NFNKAVAEAKNTGNY; peptide 14: EEPIYAQVAKKVNAK; peptide 15: AESAKKVPASLSAKL; peptide 16: TGYYCLAEENAEHGI). For the analysis of CagA-specific T cell responses, dendritic cells (DC) were enriched from MLN suspensions of TCRβ$^{-/-}$ mice by CD11c-specific immunomagnetic isolation (BD Biosciences). A total of 80,000 DC were pulsed overnight with 50 μg/ml rCagA or its fragments and cocultured with 150,000 immunomagnetically isolated MLN-derived CD4$^+$CD25$^-$ T cells. CagA peptides were added directly to the cocultures at 10 μg/ml. IFN-γ production was

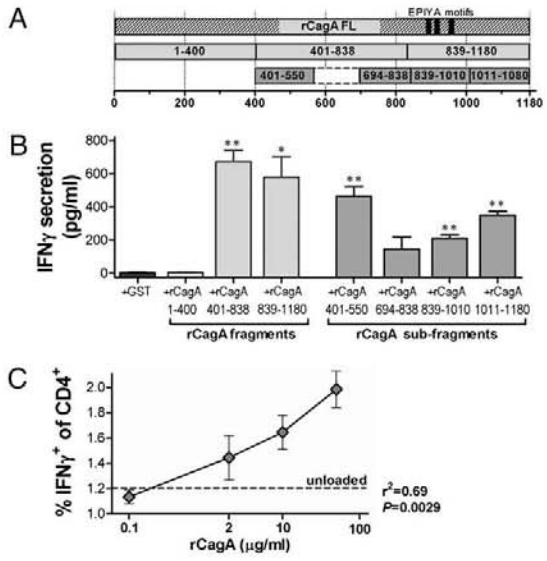


FIGURE 1. CagA harbors central and C-terminally encoded MHC class II-restricted T cell epitopes. *A*, Schematic representation of full-length CagA and the fragments used in *B*. *B* and *C*, A total of 80,000 MLN DC were pulsed with 50 μg/ml of either the GST-tag alone or the indicated CagA fragments (*B*), or with increasing concentrations of full-length rCagA (*C*), and cocultured for 4 d with 150,000 pooled CD4$^+$CD25$^-$ MLN T cells from 5 *H. pylori*-infected mice. IFN-γ secretion of cocultures was quantified by ELISA (*B*) or by intracellular cytokine staining (*C*). $^*p < 0.05$, $^{**}p < 0.01$ in relation to the GST-loaded DC. The correlation in *C* between the CagA concentration used for DC loading and the fraction of IFN-γ$^+$ cells of the total CD4$^+$ population was calculated by regression analysis; the p and r^2 values are indicated. Data are representative of three to five independent experiments.

assessed by ELISA (BD Biosciences) or by intracellular staining after 4 d of culture.

CagA ELISAs and IFN-γ real-time RT-PCR

For the evaluation of CagA-specific Ab titers, serum was diluted 1/10,000 and assessed in triplicate by ELISA on 96-well plates (Nunc, Roskilde, Denmark) precoated with 5 μg rCagA in carbonate buffer. Bound Abs were detected by HRP-coupled goat anti-mouse IgG, IgG1, or IgG2c (all from AbD Serotec, Kidlington, U.K.) Abs according to the manufacturer's recommendations. After addition of tetramethylbenzidine substrate (Sigma-Aldrich), the OD was measured at 655 nm. For real-time RT-PCR of IFN-γ, RNA was isolated from scraped gastric mucosa (antrum and corpus) using a NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany). A total of 1.5 μg total RNA was used for cDNA synthesis with Superscript Reverse Transcriptase III (Life Technologies). The resulting cDNA served as a template for real-time PCR performed with a LightCycler 480 using the SYBR green I master kit (Roche, Basel, Switzerland). Absolute values of IFN-γ expression were normalized to GAPDH expression. Primers and conditions are listed in Supplemental Table I.

Statistics

GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA) was used for statistical analyses. The significance of differences in histopathology categories was calculated by Mann–Whitney *U* test; all other indicated *p* values were calculated by Student *t* test. Linear regression analysis was performed when increasing concentrations of Ag were used in T cell priming experiments. In all scatter plot graphs, the medians are indicated by horizontal bars. In column bar graphs, standard deviations are indicated by vertical bars.

Results

H. pylori *CagA harbors MHC class II-restricted T cell epitopes in its central and C-terminal domains*

H. pylori harboring a functional T4SS triggers Th1 cell infiltration into the chronically infected gastric mucosa and the subsequent formation of atrophic, hyperplastic, and metaplastic lesions (24). We hypothesized that the only known T4SS substrate and strong humoral Ag, the CagA protein, might itself function as an MHC class II-restricted T cell Ag and trigger T cellular IFN-γ production. To test this possibility, we expressed and purified full-length CagA derived from *H. pylori* PMSS1, as well as fragments corresponding to the N-terminal, C-terminal, and central domains of the protein as N-terminally tagged GST fusions (Fig. 1*A*). Immunomagnetically isolated DC were pulsed with purified protein and cocultured with $CD4^+CD25^-$ T cells isolated from the gut-draining MLN of *H. pylori*-infected mice before the quantification of IFN-γ secretion. Full-length CagA, as well as the fragments corresponding to aa 400–838 and aa 838–1180, efficiently stimulated IFN-γ production of the cocultures; a fragment containing aa 1–400 was inactive in this regard, as was the GST tag itself (Fig. 1*B*, Supplemental Fig. 1). Further subcloning and testing of 150-aa-long subfragments revealed the existence of at least three to four distinct central and C-terminal T cell epitopes (Fig. 1*A*, 1*B*). T cells from infected mice generally responded more strongly than T cells from uninfected mice in this experimental setup (Supplemental Fig. 1). CagA did not trigger T cellular IFN-γ production in the absence of APCs (data not shown). IFN-γ production in the cultures was restricted to $CD4^+$ T cells as determined by intracellular staining, and was positively correlated with the CagA concentration used for DC loading, as determined by linear regression analysis (Fig. 1*C*). The combined results imply that the central and C-terminal domains of the CagA protein contain MHC class II-restricted T cell epitopes that stimulate $CD4^+$ T cells to produce and secrete IFN-γ.

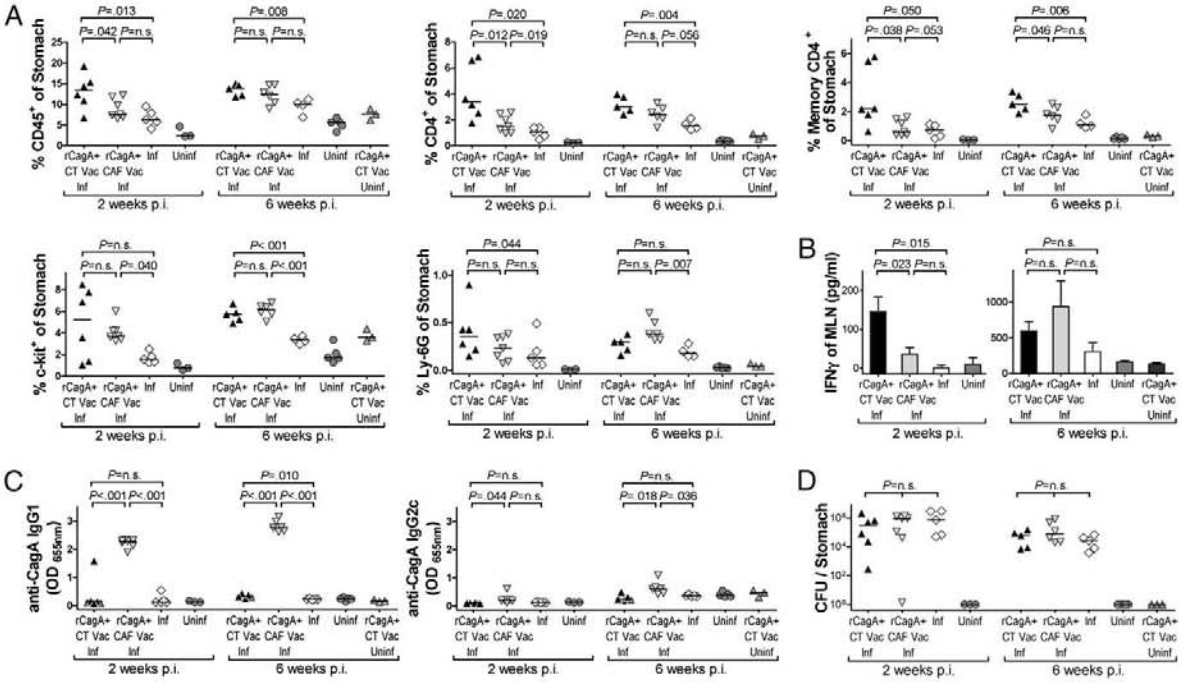


FIGURE 2. Immunization with rCagA induces local and systemic immune responses. Mice were immunized four times in weekly intervals with rCagA adjuvanted with either CT (rCagA+CT Vac) or CAF01 (rCagA+CAF Vac), challenged with live *H. pylori* together with a nonimmunized group (Inf), and sacrificed 2 and 6 wk p.i. Uninfected and immunized-only mice (rCagA+CT Vac Uninf) served as controls. *A*, Gastric infiltration of leukocytes ($CD45^+$), T cells ($CD4^+$), memory T cells ($CD44^+CD62L^-$), mast cells (c-Kit^+), and neutrophils (Ly6-G) as quantified by FACS of gastric single-cell preparations. *B*, IFN-γ secretion of MLN cultures as determined by ELISA. Averages ± SDs are shown for MLN cultures isolated from all four to seven individual mice per group. *C*, CagA-specific IgG1 and IgG2c serum titers as determined by ELISA. *D*, *H. pylori* colonization densities as determined by plating and colony counting. Data are representative of three independent immunization experiments. n.s., not significant.

Mucosal or systemic immunization with rCagA induces strong Th1-polarized responses to challenge infection but fails to confer protective immunity

To assess the immunogenicity of full-length rCagA in vivo in mucosal and systemic immunization models, we either administered CagA orally with CT or s.c. with a cationic adjuvant formulation derived from *Mycobacterium tuberculosis* (CAF01). CAF01 consists of the synthetic analog of a mycobacterial cell wall glycolipid (trehalose 6,6′-dibehenate) delivered in dimethyldioctadecylammonium liposomes; it triggers mixed Th1/Th17 responses and is successfully used in mycobacterial vaccine formulations (26). Both vaccines were administered four times at weekly intervals before challenge infection with CagA$^+$ *H. pylori*. Challenged mice were compared at 2 and 6 wk postinfection (p.i.) with nonimmunized, infected, and uninfected controls with respect to gastric infiltration of various immune cell types, T cell responses in the MLN, serum titers to CagA, and colony counts.

All immunized mice, independent of the route and adjuvant used for their vaccination, differed strongly from nonimmunized mice with respect to all parameters analyzed at both time points p.i. (Fig. 2*A–C*). CagA-vaccinated mice showed higher gastric infiltration of CD45$^+$ leukocytes, CD4$^+$ T cells, CD44$^+$CD62L$^-$ memory T cells, c-Kit$^+$ mast cells, and Ly6-G$^+$ neutrophils than infected-only controls and uninfected controls (Fig. 2*A*). *Helicobacter*-specific T cell priming and Th1 differentiation in the MLN in response to challenge infection was stronger in immunized mice as determined by IFN-γ ELISA of MLN single-cell suspension cultures of individual mice (Fig. 2*B*). A group of immunized but unchallenged mice was indistinguishable from unimmunized, uninfected controls with respect to all parameters analyzed at the 6 wk p.i. time point (Fig. 2*A*, 2*B*). High CagA-specific IgG titers were measured in the systemically immunized, but not the mucosally immunized or control infected mice (Fig. 2*C*). The IgG subclass profile further suggests a bias toward IgG1 over IgG2c production (Fig. 2*C*). The results indicate that parenteral or mucosal vaccination with rCagA enhances the cellular and humoral immune responses to challenge infection, both locally at the site of infection and in the draining lymph nodes, thereby providing evidence for the in vivo immunogenicity of CagA.

Surprisingly, however, no significant differences in colonization were observed between immunized and naive mice on challenge infection (Fig. 2*D*), indicating that immunization with adjuvanted rCagA alone does not confer protective immunity. In contrast, roughly half of all immunized mice showed widespread atrophy and hyperplasia at both 2 and 6 wk p.i., a phenomenon that is only rarely seen in infected-only controls at such early time points (Supplemental Fig. 2*A*, 2*B*). In conclusion, immunization with rCagA efficiently stimulates memory T cell responses that are reactivated on challenge infection and result in increased gastric immune cell infiltration; however, these responses fail to clear or reduce the bacteria in the vaccinated hosts.

Several distinct MHC class II-restricted T cell epitopes are encoded in the central and C-terminal domains of CagA

We next aimed to identify specific MHC class II-restricted T cell epitopes in the central and C-terminal regions of CagA. Postulating that CagA-specific T cells should be particularly abundant in CagA-vaccinated mice, and should further be enriched in the draining MLN on challenge infection, we cultured single-cell MLN preparations from individual vaccinated and control mice with rCagA. rCagA, but not the GST-tag alone, stimulated IFN-γ production by MLN cells that was stronger in the vaccinated/challenged than in the infected-only mice (Fig. 3*A*), reflecting their overall stronger reactivity to *H. pylori* (Fig. 2). MLN cultures from the same groups of mice were further restimulated in a parallel experiment with various peptides corresponding to in silico-predicted MHC class II-restricted T cell epitopes located in the central and C-terminal domains of CagA (Fig. 3*B*, 3*C*). Four of the

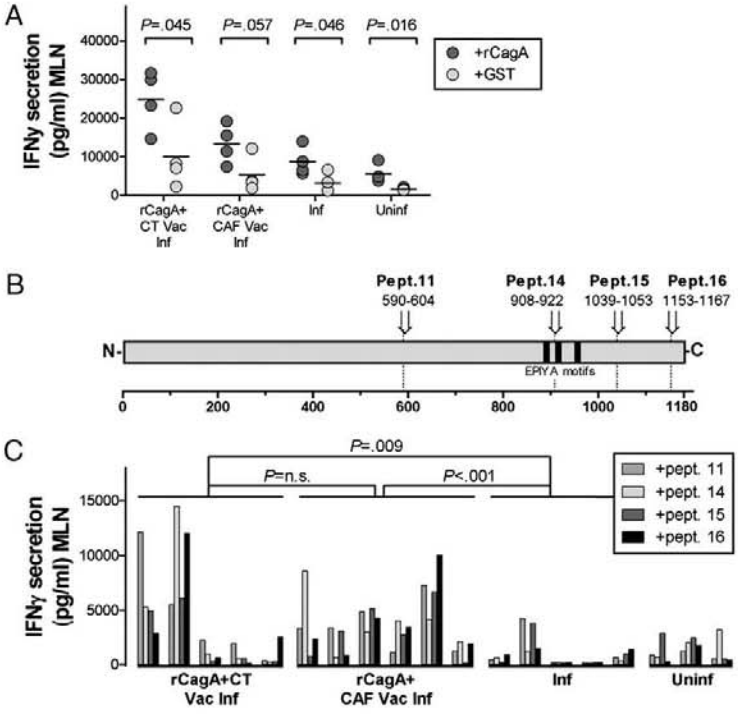


FIGURE 3. The central and C-terminal domains of CagA encode MHC class II-restricted T cell epitopes. *A–C*, MLN cultures from mice immunized and challenged as described in Fig. 2 were restimulated ex vivo for 4 d with 50 μg/ml full-length rCagA (*A*) or 10 μg/ml peptides (*C*) and assessed for IFN-γ secretion by ELISA. *B*, Schematic representation of the position of the peptides used in *C*. Data are representative of two independent experiments. n.s., not significant.

15 predicted peptides stimulated IFN-γ production in the MLN culture of at least one mouse (Fig. 3*B*, 3*C*). Most mice responded to more than one peptide; no single peptide elicited recall responses in all mice (Fig. 3*C*). Overall, the vaccinated mice of both treatment arms responded more strongly to the CagA peptides than infected-only or control mice; this reflects their overall stronger reactivity to rCagA (Fig. 3*A*). In conclusion, we show in this article that at least four distinct, nonoverlapping sequences in the C terminus of the CagA protein represent MHC class II-restricted T cell epitopes and trigger ex vivo recall responses in the draining lymph nodes.

CagA-specific T cells are sufficient to trigger gastric preneoplastic immunopathology in an adoptive transfer model

Based on our results showing that CagA-vaccinated mice respond more strongly to challenge infection than infected-only mice, and exhibit more severe gastric immunopathology, we postulated that CagA-specific T cells should be sufficient to induce gastric pathology in a host that is otherwise devoid of T cells. We used TCRβ$^{-/-}$ recipients, which cannot generate α/β^{+} T cells and are, on the one hand, incapable of controlling a *Helicobacter* infection, and on the other hand, completely protected from the T cell-driven *Helicobacter*-associated gastric immunopathology typical of immunocompetent mice (24, 27). We adoptively transferred immunomagnetically isolated, >85% pure, splenic CD4^{+}CD25^{-} T cells from CagA/CT-immunized, unchallenged mice to TCRβ$^{-/-}$ recipients, which were either experimentally infected with *H. pylori* PMSS1 on the same day or remained uninfected (Fig. 4). Additional groups of TCRβ$^{-/-}$ recipients received T cells from naive donors or from CagA/CT-immunized, challenged donors. In line with the presumed "pathogenicity" of CagA-specific T cells, the recipients of T cells isolated from CagA/CT-immunized, unchallenged mice developed extremely severe gastric pathology. This response was seen only in *H. pylori*-infected recipients; the same cells were harmless in uninfected recipients (Fig. 4*A*, 4*B*). T cells from immunized donors produced more severe pathology in infected recipients than T cells from naive donors, indicating that CagA-specific T cells must account for the infection-dependent immunopathology in the new hosts. Quite unexpectedly, the recipients of T cells from CagA-immunized mice that had been challenged with live bacteria were less pathogenic in their new hosts than cells from immunized-only mice (Fig. 4*A*, 4*B*), suggesting that an active infection in the donor suppresses T cell activity in a robust and very sustained manner. The strong pathology observed in the recipients of T cells from CagA/CT-immunized, unchallenged mice correlated with low colonization levels (Fig. 4*C*), high levels of infiltration of CD4^{+} T cells into the gastric mucosa (Fig. 4*D*), and high levels of gastric IFN-γ production (Fig. 4*E*). In contrast, the recipients of naive T cells, immunized/challenged T cells, and the uninfected recipients of immunized T cells all had relatively lower levels of gastric CD4^{+} T cell infiltration and IFN-γ production (Fig. 4*D*, 4*E*), and were colonized more heavily (Fig. 4*C*). In conclusion, the adoptive T cell transfer model revealed that CagA-specific T cells targeting the infection site in the otherwise T cell-deficient host are by themselves sufficient to produce the gastric pathology typically associated with *H. pylori* infection in immunocompetent animals, lending further support to our model that a CagA^{+} infection triggers gastric pathology through CagA's T cell antigenic properties.

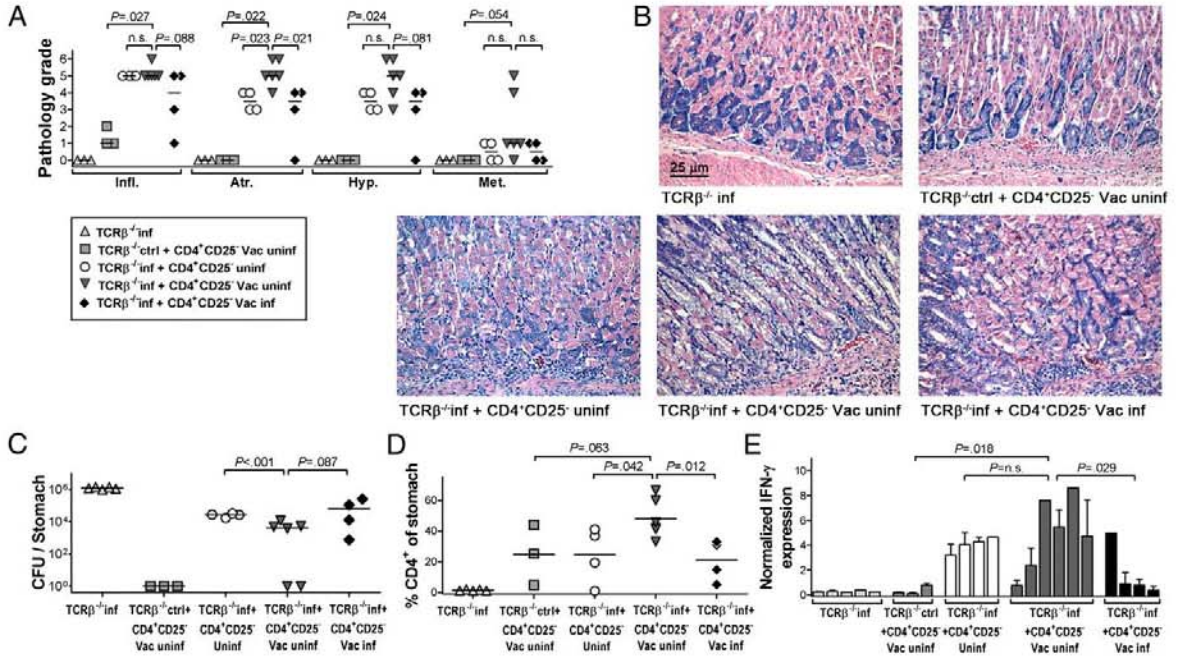


FIGURE 4. CagA-specific T cells trigger gastric preneoplastic immunopathology. Pooled splenic CD4^{+}CD25^{-} T cells were isolated from groups of mice that had been vaccinated with CagA/CT and challenged (Vac inf) or not (Vac uninf) or had remained untreated (Uninf); cells were adoptively transferred to TCR-β$^{-/-}$ recipients, which were either infected with *H. pylori* (TCR-β$^{-/-}$ inf) or remained uninfected (TCR-β$^{-/-}$ ctrl). *A–C*, Histopathology scores (*A*), representative micrographs of Giemsa-stained gastric sections (original magnification ×200) (*B*), and bacterial colonization densities (*C*) are shown for all mice 4 wk post cell transfer. *D*, Stomach-infiltrating CD4^{+} T cells as determined by FACS. *E*, Gastric IFN-γ expression as assessed by real time RT-PCR and normalized to GAPDH. *A–E*, Data are representative of two independent adoptive transfer experiments. n.s., not significant.

H. pylori-*specific vaccination promotes preneoplasia even if it confers "protective" immunity*

Vaccination with adjuvanted CagA induces local and systemic T cell responses to challenge infection that are not observed in naive mice infected for the same length of time, but nevertheless fails to confer protective immunity (Fig. 2). To assess whether a vaccination strategy conferring protective immunity sensitizes mice to enhanced gastric immunopathology, we immunized mice orally with an *H. pylori* PMSS1 whole-cell sonicate vaccine adjuvanted with CT, and challenged them with the autologous strain 2 wk after the last dose. The vaccinated mice reduced their bacterial burdens by almost two orders of magnitude compared with infected-only controls (Fig. 5*A*). However, the vaccinated mice further also developed more severe immunopathology than infected-only mice with respect to all parameters scored at 2 mo p.i. (Fig. 5*B*, 5*C*), implying that vaccination is not a suitable strategy for the prevention of gastric preneoplasia induced by $CagA^+$ strains.

Active tolerization of neonatal mice to H. pylori *prevents gastric preneoplasia*

We have shown previously that the development of immunological tolerance to *H. pylori* during the neonatal period prevents local and systemic immune responses to the pathogen and confers long-lasting protection of the host from gastritis and premalignant changes of the gastric mucosa (24). To test whether tolerization to *H. pylori* can also be achieved in the absence of active infection as an alternative strategy of gastric cancer prevention, we treated neonatal mice repeatedly with a PMSS1 whole-cell sonicate combined with anti-CD40L–neutralizing Ab during the second week of life, that is, during a time when a newborn's immune system is inherently prone to develop tolerance to self-Ags and foreign Ags (28). CD40L antagonization is used for allograft tolerization and functions by preventing costimulatory signaling during alloreactive T cell priming (29). The tolerized mice were subsequently infected as adults with live bacteria and compared with untreated mice with respect to *H. pylori* colonization, gastric T cell infiltration, and gastric histopathology (Fig. 6*A–C*). Neonatal treatment with *H. pylori* sonicate and anti-CD40L mAb was indeed sufficient to tolerize neonatal mice efficiently to the bacterium; anti-CD40L mAb-treated mice were colonized at significantly higher levels (Fig. 6*A*), exhibited lower levels of gastric T cell infiltration (Fig. 6*B*), and were protected from the gastric inflammation and associated immunopathology that are a hallmark of $CagA^+$ infection after 2 mo (Fig. 6*C*). Similar, but somewhat weaker results were obtained when rCagA was used for neonatal tolerization in conjunction with anti-CD40L mAb (Supplemental Fig. 3). Conversely, the infection-induced development of *H. pylori*-specific tolerance in neonates could be prevented by four doses of an agonistic anti-CD40 Ab administered during the first week of neonatal *H. pylori* infection; neonatally infected mice treated in this manner had significantly reduced their bacterial burdens compared with untreated, infected mice at 2 mo p.i. (Fig. 6*D*), exhibited higher levels of gastric T cell infiltration (Fig. 6*E*), and had developed significant gastritis, atrophy, and hyperplasia at this time point (Fig. 6*F*). In summary, our data suggest that the modulation of the CD40–CD40L interaction by neutralizing and/or activating Abs is a suitable method to shift the balance to either immunological tolerance or immunity, and to thereby influence disease outcome.

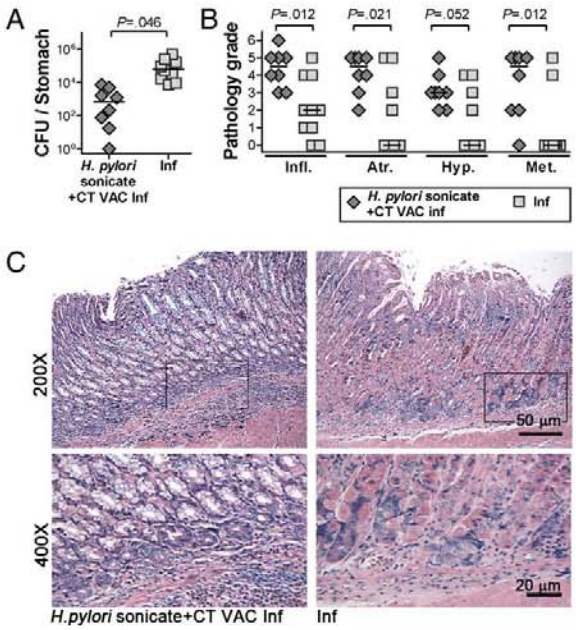


FIGURE 5. *H. pylori*-specific vaccination aggravates gastric preneoplasia. *A–C*, Mice immunized with an *H. pylori* whole-cell sonicate/CT vaccine were compared with nonimmunized counterparts 2 mo post challenge infection. *H. pylori* colonization (*A*), gastric histopathology scores (*B*), and representative low- and high-magnification micrographs of Giemsa-stained paraffin sections (*C*) are shown. Original magnification ×200 (*upper panels*) and ×400 (*lower panels*). Data are representative of three independent vaccination experiments.

Discussion

Substantial epidemiological and experimental evidence is now available to support the notion that CagA functions as a bacterial oncoprotein. A large meta-analysis of all epidemiological data available at the time showed that infection with $CagA^+$ *H. pylori* strains increases the gastric cancer risk above and beyond the risk conferred by infection alone (17). Experimental infection of Mongolian gerbils (23) and C57BL/6 mice (24) with wild type, but not CagA translocation-deficient *H. pylori* results in the rapid development of gastric cancer precursor lesions. Transgenic expression of CagA in the gastric mucosa further revealed that CagA is in itself sufficient to cause gastric hyperplasia, gastric polyps, and adenocarcinomas (22). In addition to the direct effects that the ectopic expression or natural delivery of CagA have on the cell biology of host cells, we demonstrate in this article that CagA has strong T cell antigenic properties. The central and C-terminal parts of the protein harbor in silico predicted MHC class II-restricted T cell epitopes, some of which were confirmed experimentally by ex vivo restimulation of T cells from CagA-vaccinated mice. Vaccination with CagA through two complementary routes, parenteral or mucosal, induced CagA-specific pathogenic T cells, which cause excessive gastric immunopathology in T cell-deficient recipients but fail to confer protective immunity in immunocompetent mice. Several previous findings make it seem likely that the T cell immunogenicity of CagA is at least partially responsible for CagA's oncogenic properties. First, we have shown earlier that T cells are indispensable for the induction of gastric pathology, not just in *H. pylori*, but also in *Helicobacter felis* infection models (27, 30). TCR-$\beta^{-/-}$ mice that lack functional α/β^+ T cells are protected from gastric cancer precursor lesions, and the adoptive transfer of $CD4^+CD25^-$ T cells is sufficient to trigger these lesions in resistant mice (24,

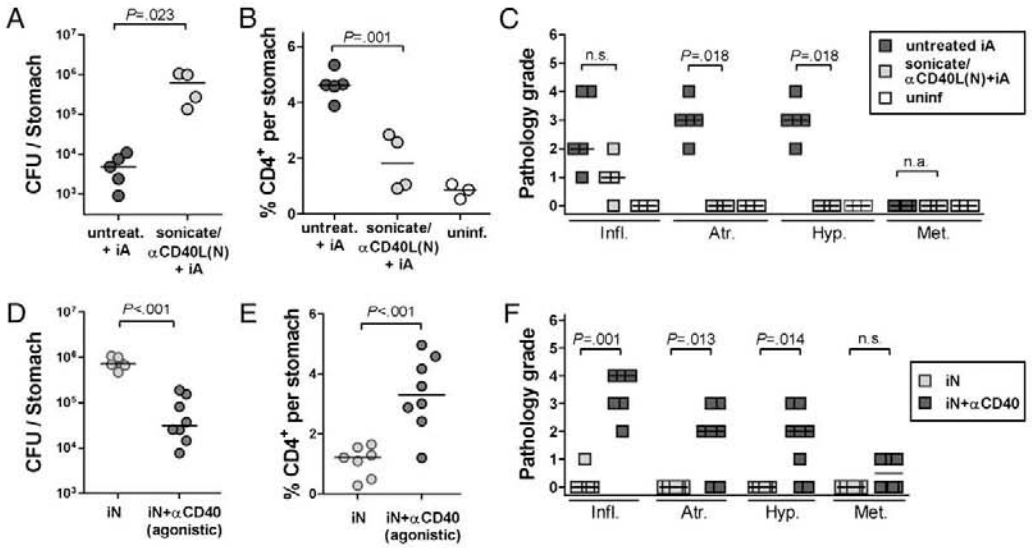


FIGURE 6. Modulation of CD40/CD40L signaling affects disease outcome. *A–C*, For the purpose of *H. pylori*-specific tolerization, neonatal mice received 4 oral doses of 50 μg *H. pylori* PMSS1 sonicate together with 50, 50, 75, and 75 μg antagonistic anti-CD40L mAb (i.p.) on days 7, 10, 12, and 14 after birth. Sonicate-treated mice and untreated control mice were infected with the autologous strain at 6 wk of age (i.e., 4 wk after the last dose) and sacrificed 8 wk later. *D–F*, Seven-day-old mice were infected with *H. pylori* PMSS1; one group received 4 i.p. doses of agonistic anti-CD40 mAb on days 0, 3, 5, and 7 p.i. (i.e., on days 7, 10, 12, and 14 after birth), whereas their littermates remained untreated. All mice were sacrificed at 8 wk p.i. *A* and *D*, *H. pylori* colonization. *B* and *E*, $CD4^+$ T cell infiltration into the gastric mucosa as determined by FACS. *C* and *F*, Histopathology scores. Uninfected, untreated control mice are shown in *B* and *C* for comparison. n.s., not significant.

27, 30). The pharmacological inhibition of T cell activation prevents and even reverses pre-existing lesions (31, 32), and neonatally infected (24) and actively tolerized mice are protected from gastric preneoplasia. Our finding that tolerant mice, despite being colonized very densely by CagA translocation-proficient bacteria, do not develop preneoplasia is perhaps the most important piece of evidence for an indirect pathogenic role of CagA (24). Finally, the results of this study provide a mechanistic explanation for our previous finding that wild type *H. pylori* and an isogenic CagE-deficient mutant differ significantly with respect to the gastric mucosal Th1 infiltration and the gastric production of IFN-γ they induce (24).

Significant progress has been made in recent years with respect to the identification of new protective *Helicobacter* Ags, understanding the mechanisms of protective immunity and the development of immunization/challenge protocols in human volunteers (33). We and others have reported previously that the challenge of whole-cell–immunized mice with avirulent mouse-adapted *H. pylori* strains such as SS1 induces more gastritis and preneoplastic lesions than infection of nonimmunized animals (27, 34, 35). In this study, we expand these findings by challenging immunized mice with a patient isolate that harbors a functional Cag pathogenicity island and is fully virulent in the murine host (24). This challenge model, which presumably mimics the human natural infection better than challenge with mouse-adapted strains, confirmed our previous results (27, 34) and demonstrated an increased susceptibility of immunized mice to gastric preneoplasia. This increased susceptibility was independent of the route of vaccine administration and of the adjuvant used, and did not correlate with a vaccination-induced reduction of the bacterial burden. Overall, the results raise the concern that any *Helicobacter* vaccine failing to achieve sterilizing immunity after challenge infection will increase rather than alleviate the vaccinee's gastric cancer risk, and will thus defy the main objective of *Helicobacter*-specific immunization.

We propose in this article that tolerization of the host to *H. pylori* may hold more promise than vaccination strategies in gastric cancer prevention. We base this proposition on our observation that neonatal mice develop natural tolerance to the bacteria if they are infected during the first 2 wk of life, at a time when the immune system is immature and inherently biased toward tolerogenic over immunogenic responses (28). Neonatally acquired, *Helicobacter*-specific tolerance is mediated and maintained by long-lived, inducible regulatory T cells, and protects the host from gastric preneoplasia not only during the neonatal period, but long into adulthood. We have expanded these findings in this study by showing that newborn mice can be tolerized actively by administration of *H. pylori* whole-cell sonicate in conjunction with anti-CD40L blocking Ab, which has been used extensively to prevent allograft rejection in preclinical models (29), and effectively prevented the gastric T cell responses and immunopathology typically associated with $CagA^+$ infection in adult-infected mice. Similar results were obtained by tolerization with CagA in conjunction with anti-CD40L Ab. The antigraft tolerization by CD40L inhibition was shown to be due to the induction of anergy in the alloreactive effector T cell pool as measured by their failure to expand and produce cytokines, but also required regulatory T cells (29). Antigraft tolerance can be broken by administration of an agonistic anti-CD40 Ab (29). Similarly, the anti-*Helicobacter* tolerance of neonatally infected mice could be broken in our hands by systemic CD40 activation.

In conclusion, we propose in this article that the increased gastric cancer risk associated with $CagA^+$ strains is due to the protein's central and C-terminally encoded T cell antigenicity. Not only do wild type *H. pylori* induce more gastric pathology than a CagA translocation-deficient isogenic mutant, but $CagA^+$ infection-associated pathology can be further exacerbated by prior immunization with rCagA. Overall, our data imply that gastric cancer management and prevention strategies will only be successful if they take into account that gastric cancer precursor lesions may be

inflammation- and/or immunity-driven rather than the direct result of bacterially induced tissue damage.

Acknowledgments

We thank Wolf-Dietrich Hardt, Jörg Hülsken, and Josef Jiricny for helpful discussions. We are grateful to Esther Kohler for expert technical assistance and to Isabella Toller, Ayca Sayi, and Vanessa Craig for assistance and discussions. We thank Wolfgang Fischer for the pGEX 4T3 expression vector and advice on CagA purification.

Disclosures

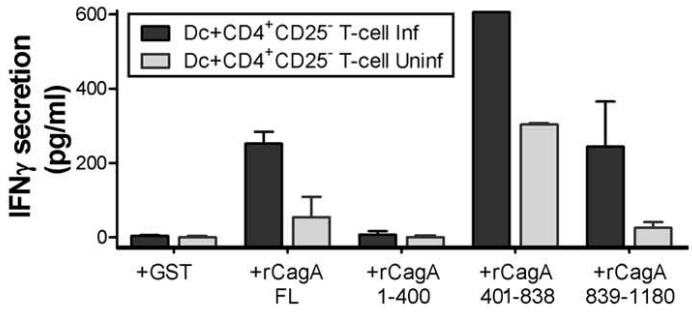
The authors have no financial conflicts of interest.

References

1. Marshall, B. J., and J. R. Warren. 1984. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet* 323: 1311–1315.
2. Parsonnet, J., G. D. Friedman, D. P. Vandersteen, Y. Chang, J. H. Vogelman, N. Orentreich, and R. K. Sibley. 1991. *Helicobacter pylori* infection and the risk of gastric carcinoma. *N. Engl. J. Med.* 325: 1127–1131.
3. Parsonnet, J., S. Hansen, L. Rodriguez, A. B. Gelb, R. A. Warnke, E. Jellum, N. Orentreich, J. H. Vogelman, and G. D. Friedman. 1994. *Helicobacter pylori* infection and gastric lymphoma. *N. Engl. J. Med.* 330: 1267–1271.
4. Pritchard, D. M., and J. E. Crabtree. 2006. *Helicobacter pylori* and gastric cancer. *Curr. Opin. Gastroenterol.* 22: 620–625.
5. Frenck, R. W., Jr., and J. Clemens. 2003. *Helicobacter* in the developing world. *Microbes Infect.* 5: 705–713.
6. Gerrits, M. M., A. H. van Vliet, E. J. Kuipers, and J. G. Kusters. 2006. *Helicobacter pylori* and antimicrobial resistance: molecular mechanisms and clinical implications. *Lancet Infect. Dis.* 6: 699–709.
7. Rupnow, M. F., R. D. Shachter, D. K. Owens, and J. Parsonnet. 2001. Quantifying the population impact of a prophylactic *Helicobacter pylori* vaccine. *Vaccine* 20: 879–885.
8. Ermak, T. H., P. J. Giannasca, R. Nichols, G. A. Myers, J. Nedrud, R. Weltzin, C. K. Lee, H. Kleanthous, and T. P. Monath. 1998. Immunization of mice with urease vaccine affords protection against *Helicobacter pylori* infection in the absence of antibodies and is mediated by MHC class II-restricted responses. *J. Exp. Med.* 188: 2277–2288.
9. Satin, B., G. Del Giudice, V. Della Bianca, S. Dusi, C. Laudanna, F. Tonello, D. Kelleher, R. Rappuoli, C. Montecucco, and F. Rossi. 2000. The neutrophil-activating protein (HP-NAP) of *Helicobacter pylori* is a protective antigen and a major virulence factor. *J. Exp. Med.* 191: 1467–1476.
10. Nyström, J., and A. M. Svennerholm. 2007. Oral immunization with HpaA affords therapeutic protective immunity against *H. pylori* that is reflected by specific mucosal immune responses. *Vaccine* 25: 2591–2598.
11. Gómez-Duarte, O. G., B. Lucas, Z. X. Yan, K. Panthel, R. Haas, and T. F. Meyer. 1998. Protection of mice against gastric colonization by *Helicobacter pylori* by single oral dose immunization with attenuated *Salmonella typhimurium* producing urease subunits A and B. *Vaccine* 16: 460–471.
12. Aebischer, T., D. Bumann, H. J. Epple, W. Metzger, T. Schneider, G. Cherepnev, A. K. Walduck, D. Kunkel, V. Moos, C. Loddenkemper, et al. 2008. Correlation of T cell response and bacterial clearance in human volunteers challenged with *Helicobacter pylori* revealed by randomised controlled vaccination with Ty21a-based *Salmonella* vaccines. *Gut* 57: 1065–1072.
13. Bumann, D., W. G. Metzger, E. Mansouri, O. Palme, M. Wendland, R. Hurwitz, G. Haas, T. Aebischer, B. U. von Specht, and T. F. Meyer. 2001. Safety and immunogenicity of live recombinant *Salmonella enterica* serovar Typhi Ty21a expressing urease A and B from *Helicobacter pylori* in human volunteers. *Vaccine* 20: 845–852.
14. Malfertheiner, P., V. Schultze, B. Rosenkranz, S. H. Kaufmann, T. Ulrichs, D. Novicki, F. Norelli, M. Contorni, S. Peppoloni, D. Berti, et al. 2008. Safety and immunogenicity of an intramuscular *Helicobacter pylori* vaccine in non-infected volunteers: a phase I study. *Gastroenterology* 135: 787–795.
15. Peek, R. M., Jr., G. G. Miller, K. T. Tham, G. I. Perez-Perez, X. Zhao, J. C. Atherton, and M. J. Blaser. 1995. Heightened inflammatory response and cytokine expression in vivo to cagA+ *Helicobacter pylori* strains. *Lab. Invest.* 73: 760–770.
16. Blaser, M. J., G. I. Perez-Perez, H. Kleanthous, T. L. Cover, R. M. Peek, P. H. Chyou, G. N. Stemmermann, and A. Nomura. 1995. Infection with *Helicobacter pylori* strains possessing cagA is associated with an increased risk of developing adenocarcinoma of the stomach. *Cancer Res.* 55: 2111–2115.
17. Huang, J. Q., G. F. Zheng, K. Sumanac, E. J. Irvine, and R. H. Hunt. 2003. Meta-analysis of the relationship between cagA seropositivity and gastric cancer. *Gastroenterology* 125: 1636–1644.
18. Odenbreit, S., J. Puls, B. Sedlmaier, E. Gerland, W. Fischer, and R. Haas. 2000. Translocation of *Helicobacter pylori* CagA into gastric epithelial cells by type IV secretion. *Science* 287: 1497–1500.
19. Segal, E. D., J. Cha, J. Lo, S. Falkow, and L. S. Tompkins. 1999. Altered states: involvement of phosphorylated CagA in the induction of host cellular growth changes by *Helicobacter pylori*. *Proc. Natl. Acad. Sci. USA* 96: 14559–14564.
20. Amieva, M. R., R. Vogelmann, A. Covacci, L. S. Tompkins, W. J. Nelson, and S. Falkow. 2003. Disruption of the epithelial apical-junctional complex by *Helicobacter pylori* CagA. *Science* 300: 1430–1434.
21. Tan, S., L. S. Tompkins, and M. R. Amieva. 2009. *Helicobacter pylori* usurps cell polarity to turn the cell surface into a replicative niche. *PLoS Pathog.* 5: e1000407.
22. Ohnishi, N., H. Yuasa, S. Tanaka, H. Sawa, M. Miura, A. Matsui, H. Higashi, M. Musashi, K. Iwabuchi, M. Suzuki, et al. 2008. Transgenic expression of *Helicobacter pylori* CagA induces gastrointestinal and hematopoietic neoplasms in mouse. *Proc. Natl. Acad. Sci. USA* 105: 1003–1008.
23. Rieder, G., J. L. Merchant, and R. Haas. 2005. *Helicobacter pylori* cag-type IV secretion system facilitates corpus colonization to induce precancerous conditions in Mongolian gerbils. *Gastroenterology* 128: 1229–1242.
24. Arnold, I. C., J. Y. Lee, M. R. Amieva, A. Roers, R. A. Flavell, T. Sparwasser, and A. Müller. 2011. Tolerance rather than immunity protects from *Helicobacter pylori*-induced gastric preneoplasia. *Gastroenterology* 140: 199–209.
25. Davidsen, J., I. Rosenkrands, D. Christensen, A. Vangala, D. Kirby, Y. Perrie, E. M. Agger, and P. Andersen. 2005. Characterization of cationic liposomes based on dimethyldioctadecylammonium and synthetic cord factor from *M. tuberculosis* (trehalose 6,6′-dibehenate)—A novel adjuvant inducing both strong CMI and antibody responses. *Biochim. Biophys. Acta.* 1718: 22–31.
26. Agger, E. M., J. P. Cassidy, J. Brady, K. S. Korsholm, C. Vingsbo-Lundberg, and P. Andersen. 2008. Adjuvant modulation of the cytokine balance in *Mycobacterium tuberculosis* subunit vaccines; immunity, pathology and protection. *Immunology* 124: 175–185.
27. Sayi, A., E. Kohler, I. Hitzler, I. Arnold, R. Schwendener, H. Rehrauer, and A. Müller. 2009. The CD4+ T cell-mediated IFN-gamma response to *Helicobacter* infection is essential for clearance and determines gastric cancer risk. *J. Immunol.* 182: 7085–7101.
28. Arnold, B., T. Schüler, and G. J. Hämmerling. 2005. Control of peripheral T-lymphocyte tolerance in neonates and adults. *Trends Immunol.* 26: 406–411.
29. Quezada, S. A., K. Bennett, B. R. Blazar, A. Y. Rudensky, S. Sakaguchi, and R. J. Noelle. 2005. Analysis of the underlying cellular mechanisms of anti-CD154-induced graft tolerance: the interplay of clonal anergy and immune regulation. *J. Immunol.* 175: 771–779.
30. Sayi, A., E. Kohler, I. M. Toller, R. A. Flavell, W. Müller, A. Roers, and A. Müller. 2011. TLR-2-activated B cells suppress *Helicobacter*-induced preneoplastic gastric immunopathology by inducing T regulatory-1 cells. *J. Immunol.* 186: 878–890.
31. Toller, I. M., M. Altmeyer, E. Kohler, M. O. Hottiger, and A. Müller. 2010. Inhibition of ADP ribosylation prevents and cures helicobacter-induced gastric preneoplasia. *Cancer Res.* 70: 5912–5922.
32. Toller, I. M., I. Hitzler, A. Sayi, and A. Mueller. 2010. Prostaglandin E2 prevents *Helicobacter*-induced gastric preneoplasia and facilitates persistent infection in a mouse model. *Gastroenterology* 138: 1455–1467, 1467.e1–e4.
33. Velin, D., and P. Michetti. 2010. Advances in vaccination against *Helicobacter pylori*. *Expert Rev. Gastroenterol. Hepatol.* 4: 157–166.
34. Mueller, A., A. Sayi, and I. Hitzler. 2009. Protective and pathogenic functions of T-cells are inseparable during the *Helicobacter*-host interaction. *Discov. Med.* 8: 68–73.
35. Sutton, P., S. J. Danon, M. Walker, L. J. Thompson, J. Wilson, T. Kosaka, and A. Lee. 2001. Post-immunisation gastritis and *Helicobacter* infection in the mouse: a long term study. *Gut* 49: 467–473.

Supplemental Figures

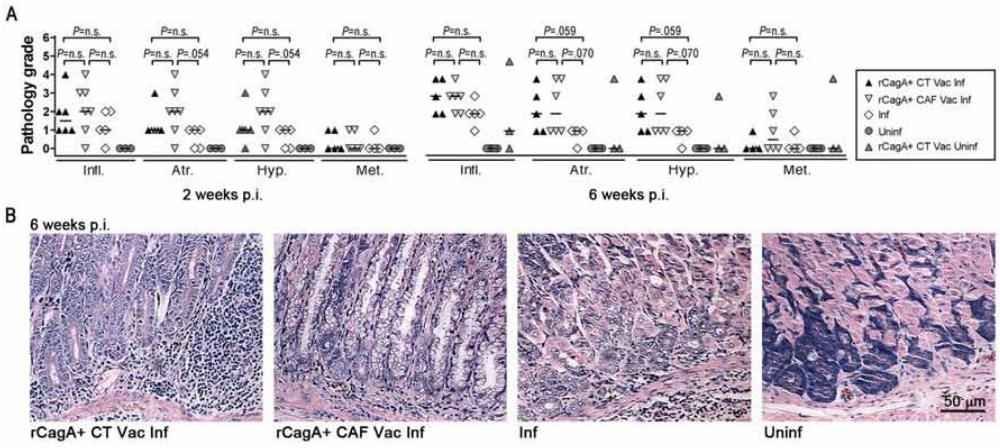
Suppl. Figure 1



Suppl. Figure 1. *Naive T-cells are less responsive to rCagA than T-cells from infected mice*

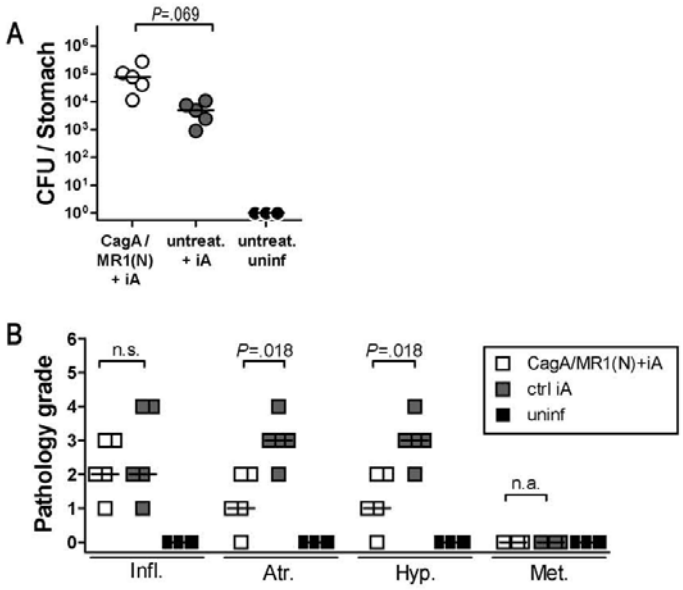
100k MLN DC were pulsed with 50µg/ml rCagA, the GST-tag alone or the indicated fragments and co-cultured for four days with 200k $CD4^+CD25^-$ MLN T-cells isolated from *H. pylori*-infected or uninfected mice. IFN-γ secretion of co-cultures was quantified by ELISA.

Suppl. Figure 2



Suppl. Figure 2. *Immunization with rCagA induces "pathogenic" rather than protective immune responses. A,B* Mice were immunized and challenged as described in main Figure 2 and assessed with respect to gastric histopathology. Scores assigned for the parameters gastric inflammation, atrophy, hyperplasia and metaplasia are shown in *A;* representative micrographs of the gastric corpus are shown in *B*.

Suppl. Figure 3



Suppl. Figure 3. *Tolerization with rCagA and anti-CD40L antibody increases H. pylori colonization and prevents gastric preneoplastic changes.* For CagA-specific tolerization, neonatal mice received 4 oral doses of 25, 25, 50 and 50µg rCagA along with 50, 50, 75 and 75µg antagonistic anti-CD40L mAb (i.p.) on days 7, 10, 12 and 14 after birth. Sonicate-treated mice and untreated control mice were infected with the autologous strain at 6 weeks of age (i.e. 4 weeks after the last dose) and sacrificed 8 weeks later. *A, H. pylori* colonization. *B,* Histopathology scores.

Supplemental Table 1

Primer sequences, annealing temperatures and cycle number of PCR reactions performed for the generation of rCagA fragments and quantitative real-time assessment of cytokine genes.

Gene	Nucleotide Sequence (5' 3')	Tm	PCR cycles
CagA FL	Fw : CAGGATCCAC TAACGAAACC ATTAACC Rv : CGGGATCCGT CGACTTAAGATTTTTGGAAACCAC	55°C	32
CagA 1-400	Fw : CAGGATCCAC TAACGAAACC ATTAACC Rv : CCGTCGACTTACTCTTTCTCGCTCAAGTTGTC	55°C	32
CagA 401- 838	Fw : CAGGATCCGACAACTTGAGCGAGAAAGAG Rv : CGGGATCCGTCGACTTAACCATTCTTAACG	55°C	32
CagA 839-1180	Fw : CAGGATCCCAATCCGTTAAGAATGGT Rv : CGGGATCCGT CGACTTAAGA TTTTTGGAAA CCAC	55°C	32
CagA 401-550	Fw : CAGGATCCGACAACTTGAGCGAGAAAGAG Rv : GATCGTCGACACTAGTGATAGCGAG	60°C	32
CagA 694 -838	Fw : CAGGATCCTGAAAGACTTTAGTAAATC Rv : CGGGATCCGTCGACTTAACCATTCTTAACG	55°C	32
CagA 839-1010	Fw : CAGGATCCCAATCCGTTAAGAATGGT Rv : GATCGTCGACTTCTGATACCGCTTG	55°C	32
CagA 1011-1180	Fw : CAGGATCCGCTAAAGCAGGTTTTTTTG Rv : CGGGATCCGT CGACTTAAGATTTTTGGAAACCAC	55°C	32
IFNγ	Fw : GGTGACATGAAAATCCTGCAGAGC Rv : TCAGCAGCGACTCCTTTTCCGCTT	58°C	35
GAPDH	Fw : GACATTGTTGCCATCAACGACC Rv : CCCGTTGATGACCAGCTTCC	55°C	32